

Proceedings of the Society for Experimental Biology and Medicine

VOL. 106

APRIL, 1961

No. 4

SECTION MEETINGS

DISTRICT OF COLUMBIA	
Georgetown University	February 2, 1961
ILLINOIS	
University of Illinois Medical School	March 14, 1961
IOWA	
State University of Iowa	January 31, 1961
MICHIGAN	
University of Michigan	January 11, 1961
SOUTHERN CALIFORNIA	
University of California, Los Angeles	January 20, 1961

Mycoplasma (PPLO) and Murphy-Sturm Lymphosarcoma.*† (26439)

E. VIRGIL HOWELL, WERNER K. OTTO AND RUSSELL S. JONES

Department of Pathology, University of Utah College of Medicine, Salt Lake City

During the regression of the Murphy-Sturm lymphosarcoma in the rat, a sporadic polyarthritis was found to be due to mycoplasma or pleuropneumonia-like organisms (PPLO)(1). These filterable microorganisms were isolated from the intact and necrotic tumor tissue as well as the joint lesions. Polyarthritis followed intravenous injection of the Mycoplasma in suitable rats. Tetracycline therapy rendered the lymphosarcoma free of PPLO and the sporadic polyarthritis no longer occurred. Jasmin(2) produced PPLO polyarthritis in rats with exudate from air pouches containing the Murphy-Sturm lymphosarcoma and demonstrated PPLO in other tumors(3). PPLO have been found in

various cell cultures *in vitro*(4,5). Because of the variable pathogenic effects of PPLO in tissues and cell cultures, it became important to evaluate the role of these microorganisms in regression of the Murphy-Sturm lymphosarcoma. The present study concerns the experimental infection of the tumor, effects of PPLO upon growth rate of the tumor and upon induced and spontaneous regression of the tumor.

Materials and methods. A PPLO-infected tumor was obtained by intravenous injection of PPLO into a rat bearing a PPLO-free Murphy-Sturm lymphosarcoma. The injected PPLO had been isolated recently from an arthritic lesion and grown in Difco PPLO broth. PPLO were cultured regularly from the subsequent transplants of the infected tumor in young, male Holtzman rats, each

* Presented in part at meeting of Am. Soc. Exp. Biol. and Med., Chicago, April, 1960.

† Supported by grant-in-aid from N.C.I., N.I.H.

weighing 100 to 120 g at time of tumor transfer. Polyarthritides did not occur during the growth of tumor. The tumor infected with PPLO and the tumor free of PPLO were transplanted by subcutaneous injection of lymphosarcoma cells as previously described (6). After the appearance of the tumors mean diameter was recorded daily and the weight derived from a plot of known diameter to mass.

In some groups of rats, cells from a PPLO-infected tumor were injected in one lumbar area and cells from a non-infected tumor in the opposite side. Rats with these double tumors were observed for "spontaneous" regression and for regression produced by *K. pneumoniae* polysaccharide (6). Both of the double tumors were cultured for PPLO. Localization of PPLO in single uninfected tumors was demonstrated by intravenous injection of recently isolated microorganisms into rats before, during and after subcutaneous transplant of tumor cells. About 12 days after tumor transplant, when the tumor had attained mean diameters of 50 to 60 mm, the rats were killed and tumor tissue cultured on PPLO-agar plates (1). PPLO colonies were detected by placing a stained cover slip upon an agar block (7). Groups of 20 or more rats with single tumors were observed for spontaneous regression of the tumor and for induction of regression by injection of polysaccharide from *K. pneumoniae*. To detect any increased susceptibility of the infected tumor, a relatively non-toxic bacterial polysaccharide was prepared by repeated alkaline extraction (8). In some groups, cortisol in an aqueous vehicle, 1 mg/100 g body weight was injected subcutaneously beginning the day of the first of 4 polysaccharide injections and continuing for 6 days thereafter. Sterile, uninoculated PPLO broth, 0.5 ml/100 g body weight, was injected into other groups of rats with PPLO-free or PPLO-infected tumors.

Results. The presence of PPLO in the tumor transplant did not alter the subsequent growth rate of the tumor (Fig. 1), increased the low incidence of spontaneous regression and doubled the regression induced by the

bacterial polysaccharide (Table 1). Even intravenous injection of uninoculated broth and presence of established PPLO polyarthritides increased the regression of the PPLO-

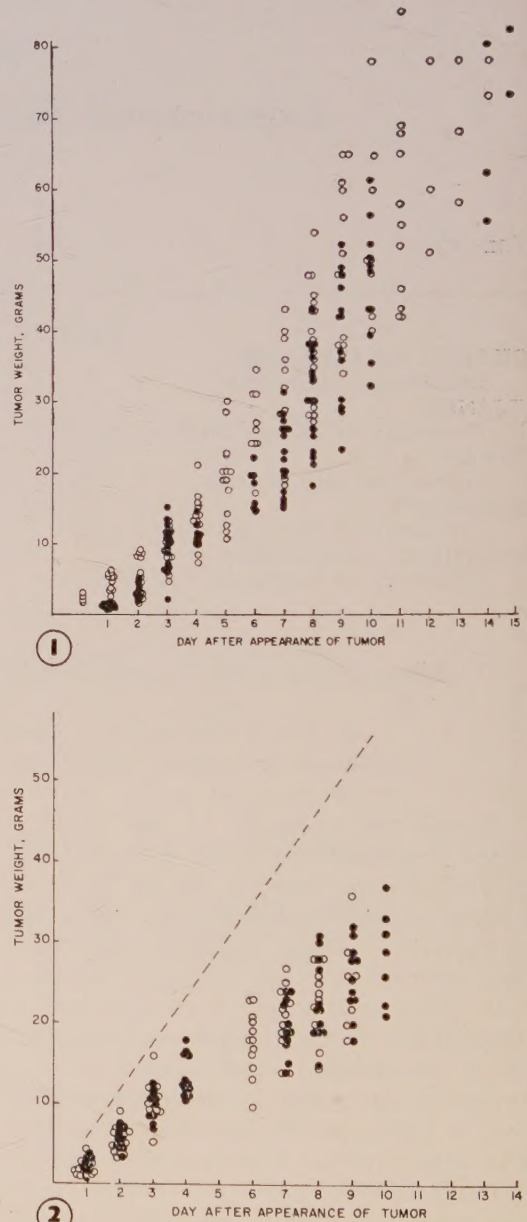


FIG. 1. Growth in representative rats of PPLO-infected (●) and PPLO-free (○) lymphosarcomas beginning at time of tumor appearance, 6 to 7 days after transplant.

FIG. 2. Similar growth rate of 2 lymphosarcomas, PPLO-infected (●), and a PPLO-free (○), in the same rat; combined avg wt of both tumors, ----.

TABLE I. Effect of PPLO in Transplanted Lymphosarcoma upon Spontaneous and Induced Regression.

Tumor	Untreated controls	Established polyarthritis*	Sterile broth†	Polysaccharide‡
PPLO-free	$\frac{6}{193}$ (3.3%)	$\frac{2}{16}$ (12.5%)	$\frac{1}{19}$ (5.3%)	$\frac{3}{14}$ (21.4%)
PPLO-infected	$\frac{10}{182}$ (5.5%)	$\frac{8}{18}$ (44.4%)	$\frac{7}{20}$ (35.0%)	$\frac{8}{17}$ (47.1%)

* Virulent PPLO inj. 11 days before tumor transplant.

† On 5th day after tumor transplant.

‡ 8th through 11th days after tumor transplant.

infected but not of the PPLO-free tumors (Table I).

When injected intravenously 3 days prior to tumor transplant, PPLO did not appear subsequently in the tumor (Table II). When PPLO were injected on day of tumor transplant, 40% of the tumors contained PPLO. All tumors examined contained PPLO when animals were injected on the 5th and 8th day after tumor transplant. Although the groups were small, the presence of PPLO in the tumor was associated with increased spontaneous regression. When PPLO were injected on the 8th day, the rapidly growing tumor (Fig. 1) led to death within the next few days before regression would be expected to occur.

In rats bearing both PPLO-infected and PPLO-free tumors, PPLO were recovered from the originally uninfected tumor after the tumor had attained large size (Table III). While growth rate of each of the double tumors was slower than that of the single tumor (Fig. 1, 2), the combined mass of the double tumors had a growth rate exceeding

that of the single tumors (Fig. 2). Both of the double tumors underwent regression simultaneously. Cortisol prevented the tumor regression produced by the bacterial polysaccharide (Table IV).

Several interesting aspects of PPLO growth were observed. Agar block preparations of PPLO-infected tumors disclosed several forms of PPLO growth, typical colonies, small colonies, "Tiny" colonies (9). Some tumor cells appeared to survive 5 to 7 days upon the special agar media. From the cytoplasmic zone of the ghost-like outlines of non-

TABLE III. Transmission of PPLO from Infected to Originally Non-infected Lymphosarcoma in the Same Rat.

Tumor at transplant	Day after transplant	
	6 to 8	12 to 13
PPLO-free	$\frac{0}{7}$	$\frac{8}{8}$
PPLO-infected	$\frac{7}{7}$	$\frac{1}{8}$ *

* All tumors contained PPLO but clonal organisms died in a few days in all agar plate cultures except one.

TABLE II. Polyarthritis, PPLO in Tumor and Tumor Regression in Rats Injected Intravenously with PPLO before, during, and after Tumor Transplant.

	Day of PPLO inj.*			
	-3	0	+5	+8
Tumor regression	$\frac{1}{10}$	$\frac{1}{10}$	$\frac{3}{10}$	$\frac{0}{10}$
Polyarthritis	$\frac{11}{20}$	$\frac{15}{27}$	$\frac{13}{18}$	$\frac{3}{15}$
PPLO in tumor†	$\frac{0}{6}$	$\frac{5}{13}$	$\frac{17}{17}$	$\frac{10}{10}$

* Tumor transplant, day 0.

† Tumor cultured at day 14.

viable cells, deeply azurophilic elements arose and penetrated the agar. These structures, superficially resembling the blue-stained PPLO, never attained large size and occurred only following the placement upon agar media of originally intact tissue or tumor cells either with or without PPLO infection. PPLO colonies usually arose from extracellular sites. In the double tumor experiment, PPLO recovered from the originally PPLO infected tumor grew a few days in clonal form then died, whereas PPLO from the originally un-

TABLE IV. Regression of 2 Lymphosarcomas (Infected and Non-infected) with PPLO in the Same Rat.

	Untreated controls	Sterile broth*	Polysaccharide†	Polysaccharide† plus cortisol
Regression	$\frac{1}{21}$	$\frac{0}{10}$	$\frac{8}{10}$	$\frac{0}{10}$
Polyarthritis	$\frac{3}{21}$	$\frac{1}{10}$	$\frac{3}{10}$	$\frac{0}{10}$

* On 11th post-transplant day.

† Beginning 11th post-transplant day.

infected tumor continued to grow on the agar plates.

Comment. In the present study PPLO transmitted with the Murphy-Sturm lymphosarcoma during transplantation, only slightly affected spontaneous regression of the tumor. However, tumors containing PPLO were more susceptible to regression upon intravenous injection of bacterial polysaccharide, sterile broth or presence of polyarthritis due to PPLO. The 2 injected materials and the polyarthritis could act through the same mechanism or mechanisms in producing tumor necrosis, with presence of PPLO in the tumor increasing its susceptibility to the substances mediating the necrosis. These observations do not afford evidence for or against several possible mechanisms of tumor necrosis such as non-specific activation of cytotoxins, stimulation of available iso-antibodies through release of materials from the tumor, or interference with critical metabolic requirements of the tumor. The presence of PPLO did not produce significant histologic or cytologic changes in the lymphosarcoma, although, grossly, such tumors showed more surrounding edema and hyperemia. These minor changes in tumor were in marked contrast to the severe chronic suppurative polyarthritis produced by PPLO isolated from the tumor and injected into normal rats.

In evaluating the role of PPLO upon the tumor several important aspects concerning PPLO were not resolved: a) the number in tumor tissue, b) intra- or extracellular location, c) virulence, d) modification of host "resistance" by large, rapidly growing tumor, and e) anti-PPLO antibody reactions. The turbidity method of counting this particular PPLO was unsatisfactory; broth cul-

tures were often non-turbid but contained the most virulent PPLO as well as numerous PPLO as estimated by placing centrifuged sediment of broth culture upon agar, staining and observing as 2000 magnification. Plate counts were considered inaccurate because of the occasional diffuse spread of organisms and variations in clonal morphology. In unpublished studies we have interconverted virulent and non-virulent PPLO *in vitro*. Upon addition of sterile tumor extract or other materials non-virulent PPLO produce polyarthritis upon intravenous injection in normal rats. Similarly, when non-virulent PPLO were injected into tumor-bearing rats, pathogenic PPLO were recovered from the tumor. These as well as Jasmin's observations(2) indicate that the Murphy-Sturm lymphosarcoma was an excellent environment for growth of pathogenic PPLO. In the present study polyarthritis was readily produced either in normal rats or rats with growing tumor by special cultural enrichment or by maintaining the PPLO in tumor tissue. In other studies, using PPLO of varying virulence, we have confirmed that rats with growing lymphosarcoma were most resistant to PPLO polyarthritis(2,10) while rats with necrotic or regressing tumors were the most susceptible(1). The failure of PPLO to appear in tumor transplanted three days after intravenous injection of PPLO may be explained by the rapid death of PPLO in animal tissues other than synovial structures. The transmission of PPLO from an infected to an originally uninfected tumor in the same animal (Table III) was in contrast to the lack of tumor infection with prior PPLO polyarthritis (Table II). Thus, on one hand, PPLO established in either intact tumor or

joint did not appear in the other tissue site, while, on the other hand, PPLO in one tumor were transmitted to another tumor. Furthermore, the varied time of appearance of separate arthritic lesions suggest non-contiguous dissemination from one joint to another. To the uncertain roles of local suppuration and of the anti-PPLO antibodies in transmission of PPLO in the above circumstances, one may add the role of tumor tissue *per se*—a remarkable milieu in which antigen-antibody and inflammatory responses, quite unlike those in other tissues, would not be unexpected.

Summary. Mycoplasma (PPLO) transmitted with the Murphy-Sturm lymphosarcoma at time of transplant, a) did not modify tumor growth, b) mildly increased the low incidence of spontaneous regression of the tumor and, c) increased the regression due to bacterial polysaccharide and to established PPLO polyarthritis. Following their intravenous injection, PPLO were readily cultured from tumor tissue. When injected prior to transplant of tumor cells, persistent

polyarthritis developed but PPLO did not appear in the subsequent tumor. However, when 2 tumors, one infected and one free of PPLO, were transplanted to the same rat, PPLO appeared in the original PPLO-free tumor after moderate tumor size was attained. Agar plate cultures of PPLO indicated several colony sizes and an occasional diffuse surface spread.

1. Howell, E. V., Ward, J. R., Jones, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1959, v102, 210.
2. Jasmin, G., Richer, C. L., *Experientia*, 1959, v15, 329.
3. Jasmin, G., *Rev. Canad. Biol.*, 1956, v15, 107.
4. Collier, L. H., *Nature*, 1957, v180, 757.
5. Hayflick, L., Stinebring, W. R., *Ann. N. Y. Acad. Sci.*, 1960, v79, 433.
6. Thomas, C. R., Jones, R. S., *Arch. Path.*, 1960, v70, 441.
7. Dienes, L., Ropes, M. W., Smith, W. E., Madoff, S., Bauer, W., *N. Eng. J. Med.*, 1948, v238, 509.
8. Jones, R. S., Carter, Y., *Arch. Path.*, 1957, v63, 484.
9. Shepard, M. C., *J. Bact.*, 1956, v71, 362.
10. Jasmin, G., *Ann. Rheum. Dis.*, 1957, v16, 365.

Received January 30, 1961. P.S.E.B.M., 1961, v106.

Comparison of Autologous, Homologous, and Heterologous Normal Skin Grafts in the Hamster Cheek Pouch.* (26440)

STANLEY N. COHEN† (Introduced by J. R. Brobeck)

Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia

During recent years, the cheek pouch of the Syrian hamster has become an increasingly popular site for transplantation of fetal (1,2), malignant(3,4,5,6), and normal adult (7) tissues. Toolan(8) has observed increased growth potentialities of normal heterologous tissues implanted into the cheek pouch of the cortisonized hamster. Lemon, *et al.*(3) have reported the persistence of some normal human tissues in the cheek pouch of untreated hamsters but have failed to observe definite evidence of growth. It

appeared desirable to evaluate carefully the cheek pouch as a site for grafted tissues and to compare the behavior of normal autologous, homologous, and heterologous grafts in both cortisonized and unconditioned hosts.

Methods. Male young adult Syrian hamsters, from a non-inbred colony, weighing between 70 and 100 g were used in all of these experiments. The animals were implanted with 1 mm square portions of normal skin according to the method of cheek pouch implantation described by Lutz, *et al.*(9). The entire thickness of abdominal skin was taken for the hamster homografts and autografts. For the heterografts, a 1 mm thickness of

* Supported in part by U.S.P.H.S. grants.

† Present address: Mount Sinai Hospital, New York.

TABLE I. Survival of Skin Grafts in Hamster Cheek Pouch.

Type of graft	Sacrificed 3 wk after implantation				Sacrificed 6 wk after implantation			
	Cortisonized		Unconditioned		Cortisonized		Unconditioned	
	No. of implants	No. surviving	No. of implants	No. surviving	No. of implants	No. surviving	No. of implants	No. surviving
Heterologous	13	10	14	7	12	9	13	6
Homologous	11	7	13	12	12	7	11	9
Autologous	12	6	11	9	14	8	12	12

adult rabbit skin freshly obtained from the back of a black-haired strain and removed from an area in the inactive phase of the hair growth cycle was employed. The skin was shaved, then wiped with dry sterile gauze before removing it. The grafts were not treated in any other way.

Bilateral implantations were employed, and all 3 possible combinations were studied—*i.e.*, auto-homo, auto-hetero, and homo-hetero. Half the recipient animals received 3 mg of cortisone acetate (Merck) subcutaneously in the nape of the neck at time of implantation and 2 mg twice weekly for the remainder of experiment. The remaining animals were untreated.

Animals were sacrificed after 3 and 6 weeks and degree of growth of the graft in the cheek pouch was determined by examination of histological specimens. The criteria employed in determining successful or unsuccessful survival were: mitotic activity, appendage formation (hair follicles, sebaceous glands), pigment formation, and migratory activity of the epidermis(10). Infection widespread enough to cause significant necrosis of the graft and/or host tissue occurred in approximately 10% of all implantations. In these instances, the section was discarded from the series. One of the 36 animals used died during the experiment and was not included in the series.

In addition to being rated microscopically as "surviving" or "rejected", sections were subjectively evaluated on a "blind" basis—using an arbitrary 0 to 4 scale—and employing degree of mitotic activity, appendage and pigment formation, and migratory activity as criteria. This procedure produced evaluations of surprising consistency over repeated trials.

Results. For 6-10 days after implantation, all grafts appeared grossly as yellowish-white nodules 3-8 mm in diameter surrounded by a variable area of increased vascularity. Surviving grafts retained this general appearance. Regressing transplants decreased in size after 6-10 days and about one-third was completely absorbed by 16-20 days. In the remaining two-thirds of regressing grafts, nodules 1-2 mm in diameter marking the site of a granulomatous reaction persisted for 2-4 weeks longer.

Survival of the 3 types of grafts is shown in Table I. Heterografts were surviving at 3 and 6 weeks in both cortisonized and untreated hamsters. The hair cycle, which was in the inactive phase at time of implantation, converted to the active phase—and mitoses, hair follicle and gland development, and pigment formation were seen in surviving grafts in both conditioned and untreated animals (Fig. 1, *top*). Epithelial migratory activity was marked (complete encystment by 3 weeks) in all surviving implants, and most of the necrotic implants showed evidence of partial encystment before being rejected. All cysts were of the "external"(11) type, with the cuticular layer facing the center of the cyst and the basal layer facing outward. (Fig. 1, *bottom*). The appearance of the grafts was not significantly different at 3 and 6 weeks. Subjective evaluation as well as survival-regression ratios indicated that heterografts were growing more successfully in cortisonized animals. There was no significant difference in the behavior of cheek pouch autografts and homografts from the same non-inbred colony (Table I). In contrast to the behavior of heterografts, both autografts and homografts grew more successfully in *non-cortisonized* animals. Indeed, there were

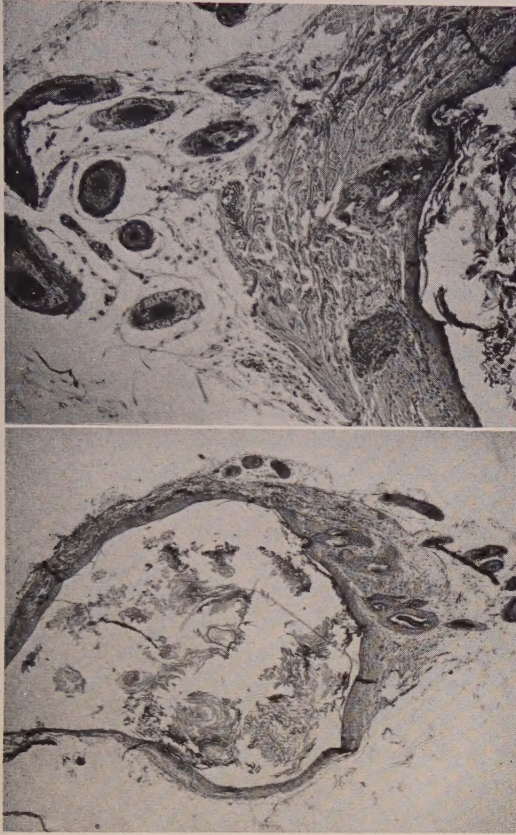


FIG. 1, top. Adult rabbit skin heterograft in cheek pouch of unconditioned hamster 42 days after implantation. $\times 72$. Development of dermal appendages and pigment formation are evident.

FIG. 1, bottom. Adult rabbit skin heterograft in cheek pouch of unconditioned hamster 42 days after implantation. $\times 30$. Complete "external" encystment has occurred via epithelial migration.

a surprisingly small number of "takes" in treated hamsters.

All rejected grafts were surrounded and infiltrated by large numbers of lymphocytes and plasma cells. In addition, smaller numbers of foreign body giant cells, monocytes, and polymorphonuclear elements were seen amidst the necrotic tissue. Lymphocytes and plasma cells in significant numbers surrounded and infiltrated 10 of the 13 surviving heterografts in untreated animals—but graft survival and growth occurred despite the presence of this infiltration. A lesser mononuclear cell accumulation was noted in surviving heterografts in cortisonized hamsters, and still fewer were seen in surviving autografts and homografts.

It should be noted that changing the kind of graft in one cheek pouch had no effect on the contralateral cheek pouch graft in the same animal. For example, heterografts grow equally well whether a homograft or an autograft was in the opposite cheek pouch.

In view of the surprising extent of heterograft survival in untreated hamsters, orthotopic heterografts of adult rabbit ear skin were placed on the flanks of 10 hamsters from the same non-inbred colony according to the method described by Billingham and Medawar(12). Four of the recipient animals received the same dose of cortisone given to cheek pouch implant recipients; the remainder were untreated.

In contrast with the results observed in the cheek pouch, these orthotopic heterografts were uniformly non-viable beyond the 7th day. Cortisone was completely ineffective in prolonging the survival of these grafts. Following initial vascularization, evident on the third or fourth day after implantation, hemorrhagic necrosis developed and the graft was rejected in the familiar fashion(12,13).

Discussion. A number of workers have reported growth of adult heterologous skin implants in the cheek pouch of the cortisonized hamster(5,3,7). Foley and Handler (3), working with tissue culture cell inocula, found that normal heterologous tissue injected in a sufficiently large inoculum will grow and proliferate for at least a short period of time in the hamster cheek pouch, even when no cortisone treatment of the recipient is employed. Billingham and Hildemann(13) have shown (and the experiments reported here have confirmed) that normal skin heterografts are promptly rejected from the body surface of the hamster in every instance—even with the use of cortisone, and have suggested that "the cheek pouch may be an immunologically privileged environment, where grafts either fail to elicit a maximal response or are to some extent exempt from its consequences." The experiments reported here (*i.e.*, survival and growth of adult skin heterografts in the hamster cheek pouch even without conditioning of the recipient) suggest that the cheek pouch *is* in some way immunologically privileged and that grafts placed

there are not subject to the same factors causing prompt rejection of orthotopic skin heterografts. These experiments suggest, however, that the sanctuary of the cheek pouch is not complete for, when heterografts are given further protection against rejection by use of cortisone, they grow more successfully than they do without the steroid.

The acceptance of intracolony orthotopic homografts by the hamster and the behavior of these like autografts has been reported by a number of investigators, and thus the survival of intracolony cheek-pouch homografts was not unexpected. It is interesting to note, however, that in contrast to heterografts, both auto and homografts demonstrated poorer survival in cortisonized animals than in untreated controls. The findings reported here are consistent with those reported by Vasiliev in rats(15), *i.e.*, that cortisone enhanced the growth of heterologous tumors but inhibited the growth of tumor homografts.

A word should be said about the marked lymphocytic response noted in *surviving* heterografts as well as in all necrotic implants. If one adheres to the current concept that lymphocytic elements are associated with the phenomenon of graft rejection, one is struck by the seeming impotence of these lymphocyte and plasma cell infiltrations amid the proliferating cheek pouch implants. This response was noted in those animals sacrificed at 3 weeks, and presumably had been present in the "6-week" group for at least 21 days without changing significantly in appearance or causing graft necrosis. It appears that a base-line lymphocytic infiltration occurs in response to all grafts (even autografts) but an increased number of these cells accumulates specifically in response to heterografts. This additional accumulation is inhibited by cortisone.

Experiments are presently underway to determine the basis of the cheek pouch's apparently privileged state.

Summary. 1) Adult rabbit-skin hetero-

grafts were capable of surviving at least 6 weeks and proliferating in cheek pouches of both cortisonized and untreated Syrian hamsters. Orthotopic heterografts were uniformly non-viable beyond the seventh day. 2) No difference in survival was observed between autografts and homografts from the same non-inbred colony. 3) A marked lymphocytic reaction was evident in all rejected grafts and in surviving heterografts. This response was inhibited by cortisone and apparently did not prevent growth of these heterografts. 4) In general, cortisone inhibited growth of homografts and autografts and enhanced growth of heterografts. 5) The nature of the graft in one cheek pouch had no effect on the growth of the contralateral graft.

I am grateful to Dr. Charles Breedis for his invaluable suggestions and advice in connection with this work.

1. Greene, H. S. N., *Cancer Research*, 1943, v3, 809.
2. Toolan, H. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v86, 607.
3. Lemon, H. M., Lutz, B. R., Pope, R., Parsons, L., Handler, A. H., Patt, D. I., *Science*, 1952, v115, 461.
4. Toolan, H. W., *Cancer Research*, 1953, v13, 389.
5. Handler, A. H., *Proc. Am. Assn. Cancer Research*, 1956, v2, 114.
6. Toolan, H. W., *Cancer Research*, 1954, v14, 660.
7. Hambrick, G. W., Jr., Bloomberg, R., *J. Invest. Derm.*, 1957, v29, 353.
8. Toolan, H. W., *Cancer Research*, 1957, v17, 248.
9. Lutz, B. R., Fulton, G. P., Patt, D. I., Handler, A. H., Stevens, D. F., *ibid.*, 1951, v11, 64.
10. Medawar, P. B., *Quart. J. Micros. Sci.*, 1948, v89, 239.
11. ———, *J. Exp. Path.*, 1948, v29, 58.
12. Billingham, R. E., Medawar, P. B., *J. Exp. Biol.*, 1951, v28, 385.
13. Billingham, R. E., Hildemann, W. H., *Ann. N. Y. Acad. Sci.*, 1958, v73, 676.
14. Foley, G. E., Handler, A. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1957, v94, 661.
15. Vasiliev, U. M., *Prob. Oncol.*, 1958, v4, 9.

Received February 1, 1961. P.S.E.B.M., 1961, v106.

Failure of Benzydrolumethiazide and Hydrochlorothiazide to Influence Plasma Potassium in Nephrectomized Dogs.* (26441)

WILLIAM P. BLACKMORE

Department of Pharmacology, University of Texas Southwestern Medical School, Dallas

The pharmacological basis of the hypotensive action of chlorothiazide and structurally related compounds continues to be a controversial problem. Evidence from several laboratories(1,2,3,4,5) indicates that the initial antihypertensive response of chlorothiazide is a result of reduction in plasma volume and consequent oligemia. These results are further supported by observation that restoration of plasma volume by intravenous dextran solutions, either salt free or salt containing, results in partial reversal of the antihypertensive action of chlorothiazide(6,7). In addition, chlorothiazide and mercurial diuretics have been reported to have a selective antihypertensive action which may be potentiated by a reduction in body sodium(8). The foregoing results strongly suggest that the principal action of the benzothiadiazine diuretics is dependent upon a reduction in plasma volume, resulting from the diuretic action of the drug. In contrast, it has been reported that the hypotensive response of chlorothiazide *per se* may depend upon antagonism to an unknown pressor substance (9). Chlorothiazide has also been shown to decrease "reactivity" to various types of pressor agents in normotensive and hypertensive subjects(4,10) as well as in normotensive dogs(11). This response is not specific for chlorothiazide since a similar effect concomitant with diuretic action has been observed with acetazolamide and mercaptomerin, indicating sodium excretion played a dominant role(12). Recently it has been reported that chlorothiazide and hydrochlorothiazide[†] significantly lowered plasma potassium levels with no effect on sodium levels in nephrectomized dogs(13) whereas, acetazolamide and mercaptomerin had no effect. These results suggest that the chlorothiazide

derivatives have an extrarenal action and focus attention on both an extrarenal effect and a saluretic response contributing to the early hypotensive action observed with these diuretic agents, and that they exhibit a specific effect not shared by other diuretics.

During an investigation of benzydrolumethiazide[‡] it seemed of interest to determine the effect of this diuretic on plasma sodium and potassium levels in acute nephrectomized dogs. It would be anticipated *a priori* that this structurally related agent would decrease plasma potassium if the extrarenal effect is specific for the benzothiadiazine derivatives. Our results indicate that benzydrolumethiazide does not alter plasma potassium in acute nephrectomized dogs.

Methods. Experiments were carried out with dogs of either sex weighing 9 to 20 kg that had been maintained on a diet of Friskies dog cubes for a minimum of 1 week prior to experiment. The animals were anesthetized with sodium pentobarbital (35 mg/kg) and arterial blood pressure was recorded with a 3 channel physiograph. Acute bilateral nephrectomies were performed *via* the ventral approach. Blood samples were obtained before nephrectomy(C), 1 hour post nephrectomy (PN-1 hr) immediately before administration of the diuretic, then 30 minutes (PD-30 min), 1 hour (PD-1 hr), 1.5 hours (PD-1.5 hr), 2 hours (PD-2 hr), 3 hours (PD-3 hr) and 4 hours (PD-4 hr) after beginning of drug administration. Blood hematocrits were determined and plasma sodium and potassium were measured with a Beckman flame photometer. A control group of 8 dogs received 10 ml 5% glucose in water 1 hour after nephrectomy followed immediately with a 30 minute infusion of 50 ml 5% glucose. In the control animals the 30 minute sample was not taken. All experimental animals received the same amount of glucose plus the diuretic that was being in-

* Supported by grants from U.S.P.H.S. and E. R. Squibb & Sons.

† Hydrodiuril supplied by Merck, Sharp and Dohme, Inc.

‡ Naturetin supplied by E. R. Squibb & Sons.

TABLE I. Plasma Electrolytes Values in Acute Nephrectomized Dogs.

Group and No. animals	C	PN-1 hr	PD-30 min.	PD-1 hr	PD-1.5 hr	PD-2 hr	PD-3 hr	PD-4 hr
Na, meq/l								
Control	140 \pm 1.9	139 \pm 1.9		138 \pm 1.3	140 \pm 1.6	139 \pm 2.0	139 \pm 2.0	140 \pm 1.0
Benzydoflumethiazide 12	141 \pm 1.7	141 \pm 1.7	140 \pm 1.4	140 \pm 2.2	141 \pm 1.0	142 \pm 1.7	141 \pm 1.3	140 \pm .9
Hydrochlorothiazide 12	141 \pm 1.8	140 \pm 1.4	140 \pm 1.9	141 \pm 1.5	141 \pm 1.6	142 \pm 1.6	143 \pm 1.7	142 \pm 1.5
K, meq/l								
Control 8	4.1 \pm .10	4.0 \pm .14		4.0 \pm .11	3.9 \pm .10	3.9 \pm .13	3.9 \pm .14	4.0 \pm .08

vestigated. The second group of 12 animals received a prime dose of 0.5 mg/kg benzydoflumethiazide and the same dose of the drug during the 30 minute infusion. The first 6 animals that received benzydoflumethiazide were followed for only a period of 2 hours after nephrectomy, to mimic the procedure employed for hydrochlorothiazide by Beavers (13). Thereafter all experiments were extended from 2 to 4 hours and more blood samples obtained. The third group of animals received 2 mg/kg hydrochlorothiazide as a prime dose and the same amount in the infusion. Statistical analysis of the data was performed by the method of Mather(14).

Results. Table I summarizes plasma sodium levels in control animals and those administered benzydoflumethiazide and hydrochlorothiazide as well as plasma potassium levels in control animals. Tables II and III illustrate the effect of benzydoflumethiazide and hydrochlorothiazide, respectively, on plasma potassium levels. Data for potas-

sium are presented in detail because they do not confirm previous data(13). There was no significant change in plasma sodium concentration after either diuretic ($P>0.05$). Similarly, no significant change in plasma potassium was observed in animals that received either diuretic ($P>0.05$).

Mean arterial blood pressure did not decrease more than 20 mm Hg after nephrectomy. Hematocrits for each group did not vary more than 10% from control values throughout the experiments.

Discussion. Results obtained in this study indicate that benzydoflumethiazide does not have any effect on plasma sodium and potassium levels in the acute nephrectomized dog. These results do not support the concept of an immediate extrarenal action which has been suggested for this type of diuretic(13). However, these acute experiments do not rule out the possibility that chronic administration of the drug may exhibit some extrarenal effect. Failure to observe a decrease in

TABLE II. Plasma Potassium Values in Acute Nephrectomized Dogs During and Following Administration of Benzydoflumethiazide.

Dog	C	PN-1 hr	PD-30 min.	PD-1 hr	PD-1.5 hr	PD-2 hr	PD-3 hr	PD-4 hr
1	3.7	3.7		3.7		3.7		
2	5.0	4.7		4.7		4.7		
3	4.7	4.6		4.2		4.4		
4	4.8	4.8		4.4		4.4		
5	3.7	4.0		3.7		3.7		
6	4.0	4.6		4.7		4.6		
7	3.9	4.0	3.7	4.0	4.3	4.1	4.0	4.4
8	3.6	3.6	3.8	3.5	3.7	3.9	3.9	3.6
9	4.3	4.1	3.9	4.2	4.0	4.2	4.2	4.4
10	4.5	4.7	4.8	4.4	4.3	4.6	4.5	4.4
11	3.8	3.7	4.1	4.0	3.6	3.7	4.0	4.1
12	4.2	3.8	3.9	4.1	3.7	3.9	3.9	3.9
Mean & S.E.	4.1 \pm .14	4.2 \pm .13	4.0 \pm .16	4.1 \pm .11	3.9 \pm .12	4.2 \pm .11	4.1 \pm .10	4.1 \pm .13

TABLE III. Plasma Potassium Values in Acute Nephrectomized Dogs During and Following Administration of Hydrochlorothiazide.

Dog	C	PN-1 hr	PD-30 min.	PD-1 hr	PD-1.5 hr	PD-2 hr	PD-3 hr	PD-4 hr
1	4.7	4.6	4.6	4.5	4.6	4.7	4.9	4.4
2	3.8	3.8	3.4	3.6	3.9	4.0	4.2	4.0
3	3.7	3.5	3.6	3.8	3.7	3.7	4.2	4.2
4	3.8	3.9	3.7	3.5	3.7	4.3	4.0	3.8
5	4.1	4.2	4.1	4.5	3.9	4.2	4.1	4.4
6	3.8	3.7	3.8	4.0	4.2	4.2	4.2	4.2
7	3.8	3.5	4.1	3.8	3.7	4.0	3.5	3.5
8	4.1	3.8	3.8	3.6	4.0	4.0	4.3	4.4
9	4.2	3.8	3.8	4.3	4.2	3.9	4.3	4.1
10	3.8	3.7	3.9	3.6	3.8	3.8	3.9	3.9
11	3.6	3.6	3.7	3.8	3.8	3.7	3.7	3.8
12	3.9	4.0	3.9	3.9	4.1	4.1	4.2	4.0
Mean & S.E.	3.9 ±.11	3.8 ±.09	3.9 ±.09	3.9 ±.10	4.0 ±.08	4.1 ±.08	4.1 ±.10	4.1 ±.08

plasma potassium cannot be explained on insufficient dosage since benzydrolumethiazide is at least 5 to 10 times as potent as hydrochlorothiazide in producing a saluretic effect. The only conclusion that can be obtained from this study is that benzydrolumethiazide along with acetazolamide and mercaptomerin exhibits no extrarenal action when evaluated in acutely nephrectomized dogs.

The cause of the failure to obtain a decrease in plasma potassium with hydrochlorothiazide remains obscure. The data do not show any trend toward a decrease in potassium after nephrectomy. Extending the period of observation from 2 to 4 hours should have made it more apparent if there was a migration of potassium from the plasma; this did not occur. Therefore, results obtained in this study indicate that hydrochlorothiazide does not cause any extrarenal effect in acute experiments under the conditions described.

Summary. Benzydrolumethiazide and hydrochlorothiazide did not have any effect on plasma sodium or potassium levels when administered intravenously to acute nephrectomized dogs. These results indicate that under the conditions described benzydrolumethiazide and hydrochlorothiazide do not have

an extrarenal effect on sodium or potassium which could explain the mechanism of their antihypertensive action.

1. Freis, E. D., Wanko, A., Wilson, I. M., Parrish, A. E., *Ann. N. Y. Acad. Sci.*, 1958, v71, 450.
2. Tapia, F. A., Dustan, H. P., Schneckloth, R. S., Corcoran, A. C., Page, I. H., *Lancet*, 1957, v2, 831.
3. Dollery, C. T., Harington, M., Kaufmann, G., *ibid.*, 1959, v1, 1215.
4. Freis, E. D., Wanko, A., Schnaper, H. W., Frohlich, E. D., *J. Clin. Invest.*, 1960, v39, 1277.
5. Conway, J., Lauwers, P., *Circulation*, 1960, v21, 21.
6. Freis, E. D., *Hypertension*, F. R. Skeleton, Ed., New York Am. Heart Assn., 1959, v7, 9.
7. Wilson, I. M., Freis, E. D., *Circulation*, 1951, v20, 1028.
8. Hollander, W., Chobanian, A. V., *J. Clin. Invest.*, 1958, v37, 902.
9. Wilkins, R. W., Hollander, W., Chobanian, A. V., *Ann. N. Y. Acad. Sci.*, 1958, v71, 465.
10. Merrill, J. P., Guinand-Baldo, A., Geordano, C., *Clin. Res.*, 1958, v6, 230.
11. Beavers, W. R., Blackmore, W. P., *Proc. Soc. Exp. Biol. and Med.*, 1958, v98, 133.
12. Blackmore, W. P., Beavers, W. R., *ibid.*, 1959, v101, 128.
13. Beavers, W. R., *ibid.*, 1960, v103, 711.
14. Mather, K., *Statistical Analysis in Biology*, Interscience Publishers, Inc., New York, 1945.

Received February 2, 1961. P.S.E.B.M., 1961, v106.

Role of Triiodothyronine-I¹³¹ Purity in T-3 Tests. (26442)

NORMAN D. LEE AND VINCENT J. PILEGGI (Introduced by R. J. Henry)
Radioisotope and Iodine Sections, Bio-Science Laboratories, Los Angeles, Calif.

This laboratory has been concerned with investigation of various thyrodiagnostic procedures based on *in vitro* partitioning of I¹³¹-labeled triiodothyronine (T-3) between plasma or serum proteins and such formed elements as erythrocytes(1) or ion-exchange resin beads(2). A number of variables have been reported as affecting reproducibility(3, 4). In a series of studies designed to evaluate other factors responsible for day-to-day variation attention was focused on the T-3 preparation. This report is concerned with purity and stability of such preparations and their role in T-3 tests.

Methods. T-3 preparations were stored in the refrigerator. Working solutions were used in a standard partitioning test employing whole blood(1). Descending chromatography was carried out using Whatman #3 paper and 2-butanol:4% NH₄OH (3:1) as solvent. Prior to spotting the T-3 preparation was diluted with a carrier solution containing a mixture of non-radioactive iodoamino acids and iodide. Strips were dried after development and surveyed with a radiochromatogram scanner (Scanogram II, Atomic Accessories) and the proportions of radioactive components were determined by planimetry. Identification of components was based on coincidence of radioactivity with spots shown by spraying the strips with a ceric-arsenious acid reagent and counterstaining with methylene blue(5).

Results. Table I shows uptake values for 4 different whole blood specimens in the standard partitioning test when T-3 from 3 different commercial suppliers was used. When compared to normal values(1), these were interpreted as consistent with hypo-

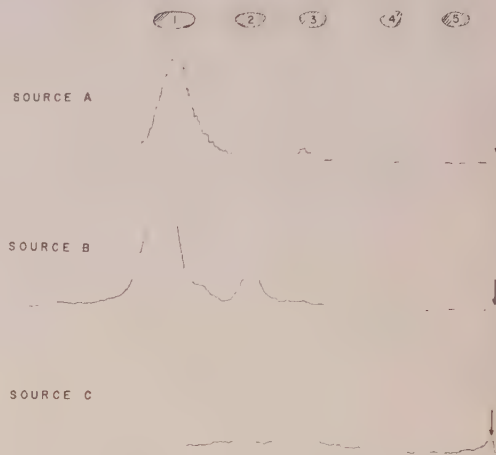


FIG. 1. Radioactive scanning records of chromatographic analysis of T-3 preparation from 3 different sources. Numbered spots at top are from a ceric-arsenious acid stained chromatogram of a mixture of iodoamino acids. (1, triiodothyronine; 2, thyroxine; 3, iodide; 4, monoiodotyrosine, and 5, diiodotyrosine. Arrows mark origin of each strip.)

eu-, or hyperthyroidism, depending on which stock T-3 preparation had been used. It could only be concluded that these differences arose from heterogeneity of the radioactive materials. This was demonstrated chromatographically; scanning records of radiochromatograms are shown in Fig. 1.

These findings prompted chromatographic analysis of a number of samples obtained from Source A; results are presented in Table II. These samples were all reasonably new. Excepting preparation #4 they can be seen to be highly uniform, being only minimally contaminated with radioactive materials other than T-3. Non-iodide impurities were also rather uniform, being distinguishable only as a deformity in one shoulder of the T-3 peak. Preparation #4 had been delayed in transit, hence its increased proportions of impurities may have arisen during this period.

Another factor is appearance of radioactive impurities with age. Data presented in Table III are concerned with 3 preparations

TABLE I. Role of Different T-3 Preparations.

T-3 from source	% uptake/100 hematocrit			
	Blood 1	Blood 2	Blood 3	Blood 4
A	17.2	17.0	14.1	13.3
B	13.8	14.3	8.8	10.4
C	6.7	6.9	4.5	5.5

TABLE II. Composition of Various Fresh T-3 Preparations.

T-3 prep. No.	Age, days	I^{131} distribution, %		
		T-3	I-	Other constituents
1	0	98	2	None
2	1	100	0	Less than 1%
3	3	100	0	<i>Idem</i>
4	4	88	4	8%
5	4	98	2	Less than 1%

which had minimal impurities on receipt. Although kept under identical conditions quantitative aspects of development of impurities seemed to be distinctly different. Time intervals employed were within or only slightly beyond the accepted usable life of such radioactive preparations.

Discussion. It was observed that there were marked differences in radiopurity of T-3 preparations obtained from different sources, that markedly impure preparations were obtained occasionally from sources having a record of high purity, and that some pure preparations developed significant proportions of impurities over a short time interval. Only the first of these problems may be directly traceable to source of supply; time and

TABLE III. Effect of Preparation Age on Proportions of Various I^{131} Constituents.

T-3 prep. No.	Age, days	I^{131} distribution, %		
		T-3	I-	Other constituents
1	0	98	2	None
	6	96	4	Less than 1%
	18	91	2	7%
3	3	100	0	Less than 1%
	20	85	3	12%
2	1	100	0	Less than 1%
	6	90	1	9%

temperature of shipment, particularly where unusual delays are involved, may be responsible for the second and third observations. Nevertheless, these phenomena certainly explain the findings shown in Table I and may account for spurious results occasionally experienced with this type of test. Chromatographic monitoring of each lot of T-3 on receipt may be a sufficiently simple procedure for routine practice. We have found use of a standard lyophilized or pooled serum of additional value as a monitoring device for each run. This is possible with any modification of the T-3 test using only patient's serum or plasma and an adsorbent other than patient's own erythrocytes.

Summary. Using a standard thyrodiagnostic test based on *in vitro* partitioning of triiodothyronine- I^{131} between plasma proteins and erythrocytes of whole blood, results were found to differ depending on the commercial source of labeled triiodothyronine. Chromatographic study showed these preparations to be heterogeneous. Impurities were occasionally present in fresh materials and developed in significant proportions during the accepted usable life of the preparation.

1. Hamolsky, M. W., Golodetz, A., Freedberg, A. S., *J. Clin. Endocrin. and Metab.*, 1959, v19, 103.
2. Sterling, K., Tabachnick, M., *ibid.*, 1961, April, in press.
3. Meade, R. C., *ibid.*, 1960, v20, 480.
4. Adams, R., Specht, N., Woodward, I., *ibid.*, 1960, v20, 1366.
5. Mandl, R. H., Block, R. J., *Arch. Biochem. Biophys.*, 1959, v81, 25.

Received February 8, 1961. P.S.E.B.M., 1961, v106.

Fluorescent Antibody Stainability and Other Consequences of the Disruption of Mycobacteria. (26443)

CHARLES C. SHEPARD AND DAVID KIRSH

Communicable Disease Center, Public Health Service, U. S. Department of HEW, Atlanta, Ga.

During the last few years a number of attempts in this laboratory to stain mycobacteria with fluorescent antibody have been unsuccessful, and the lack of publication of suc-

cessful efforts by others suggests a similar experience elsewhere.

The mycobacteria are characterized by a high lipid content and their lipophilic be-

havior(1) indicates that lipid is located on the surface. Yet water soluble preparations, usually in the form of culture filtrates, are able to react with immune serum in complement fixation and immunodiffusion reactions. It seemed possible that it was only the outer surface of the cell wall that consisted of lipid, and that the inner surface might have a structure resembling other bacteria and would contain protein or carbohydrate antigens.

To test this proposition we have disrupted mycobacteria by vibration with glass beads in the manner used successfully to prepare cell walls of many nonacid-fast bacteria (see review of Salton, 2). It was found that the mycobacteria do stain very well by fluorescent antibody after such treatment. Disruption of the mycobacteria was accompanied by a release of soluble protoplasmic constituents. This soluble fraction contains the largest amount of antigen, as judged by its inhibition of the fluorescent antibody reaction.

Vibration with glass beads had also been used by Ribi *et al.* (3) to prepare suspensions of cell walls, as judged by electron microscopy. They observed a pronounced aggregation of cell walls and unbroken organisms with consequent low yield of suspendable cell walls, and later turned to the pressure cell technic(4), where the aggregation was less severe. Clumping has been observed in the present study, but it did not hinder the observation of the markedly increased staining by fluorescent antibody. The efficiency of mycobacterial disruption was controlled by measurements of the release of soluble protoplasmic constituents.

Materials and methods. Mycobacterial cultures were maintained on Loewenstein-Jensen medium. Suspensions for study were prepared from growth in Tween-albumin medium (TB broth base of Difco), washed in water 1 to 3 times, and resuspended in water to a concentration that when diluted 10 times gave an optical density of 0.40 in the Coleman spectrophotometer with a 19 mm cuvette at 600 $m\mu$ ($D_{600} = 4.0$). The vibration was performed in the Mickle apparatus with an amplitude of 7 mm, with 4 ml of bacterial suspension and 4 g of glass beads (No. 12, Cataphote Corp., Toledo, Ohio). The sedi-

ments and supernates were collected after centrifugation at 1000 g in an angle head for 30 minutes. Bacteria were counted in microdrops by the "rapid method" (5,6), the final dilution (10^{-3}) being made in formol-milk containing 0.1% gelatin. Dry weights were performed after drying for 24 hours at 105°. The accuracy of the dry weight determinations was restricted by the availability of material but it was attempted to weigh at least 4 mg of solids in duplicate; the duplicates agreed within $\pm 10\%$. Protein was measured by the Lowry method (7). Carbohydrates were estimated by the anthrone procedure (8). An estimate (called D_{265}^{PCA}) of the amount of bacterial protoplasm was formed by measuring optical density at 265 $m\mu$ of extracts with hot perchloric acid. Four-tenths ml of bacterial preparation was mixed with 3.2 ml H_2O + 0.4 ml 60% perchloric acid and heated to 90° for 15 minutes. Centrifugation at 1000 g for 30 minutes was used to clarify the extract. The presence of Tween in the extracted material gives a turbid extract, which can be clarified by ether extraction overnight. Optical density was read in the Beckman spectrophotometer in 1 cm cells at 220, 230, 240, 260, 265, 280, 300, and 320 $m\mu$, and the curve constructed to ensure its typical form. The minimum was located at 230 or 240 $m\mu$, and the ratio between optical density at the minimum and at the maximum (265 $m\mu$) was less than 0.7 in all instances except in the case of sediments of well disrupted organisms, which had very low optical densities. This procedure does not differentiate between RNA, DNA, and smaller compounds, but is a convenient and sensitive indicator of bacterial protoplasm, as distinguished from cell wall material.

Antimycobacterial sera were prepared usually by intravenous injection of rabbits with about 0.4 mg (dry weight) of bacilli at weekly intervals for 4 weeks, with bleedings before injection and 2 weeks after 4th injection. Complement fixation tests showed antibody responses usually to titers of 1:256 to 1:2048 from levels usually less than 1:10 before immunization. Antibody response was also revealed by fluorescent antibody results.

Tubercle bacilli (H37Rv) grown 7 days in 800 ml Tween-albumin medium, washed 3 times in H₂O and concentrated to 30 ml to give $D_{600} = 4.1$

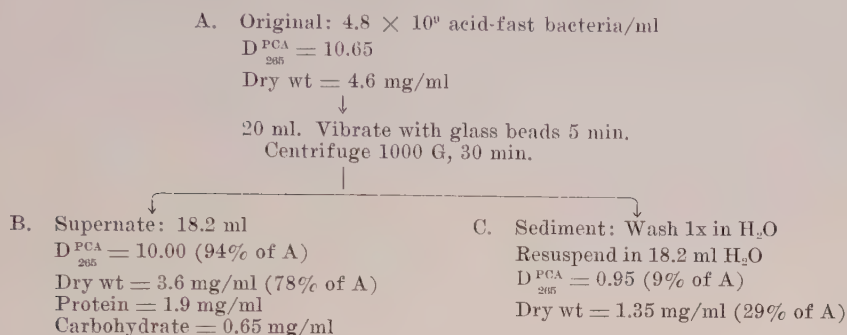


FIG. 1. Procedure for separation of intact bacilli (A) into soluble fraction (B), and disrupted organisms (C).

Most of the fluorescent antibody stains were performed by the indirect procedure with the anti-rabbit conjugates prepared from chicken or goat immune sera. Direct stains were also effective. The serum and conjugates were diluted in VBA (2.5% bovine plasma albumin in veronal buffered saline; final pH 7.5) before being applied to the slide. A fresh pipette was used for each dilution. Incubation was at 37° for 30 minutes in each case.

Labelling was performed by adding fluorescein isothiocyanate (chromatographically pure isomer I) to 20 times its weight of the globulin fraction, present as a chilled and stirring 2% solution in 0.025 M Na₂CO₃ + 0.025M NaHCO₃ + 0.15 M NaCl. After 6-18 hours the conjugates were dialyzed thoroughly and absorbed 3 or 4 times with tissue powders (prepared usually from monkey liver). The illumination for microscopy, provided by a Reichert assembly carrying an HBO-200 Osram lamp, was filtered through a half-thickness Corning 5840 filter. A Wratten 2B filter was in the ocular. With this filter system the blue-gray autofluorescence of unstained bacilli, distinctly visible in clumps of organisms, could be easily distinguished from the yellow-green fluorescence of the bacilli after they had been stained with fluorescent antibody. The smears were usually prepared fresh for each day's work. They were air-dried, fixed with acetone for 10 minutes at room temperature, then allowed to dry at room temperature. They could be preserved at -20° or lower if, be-

fore use, they were allowed to come to room temperature in a closed box to prevent condensation. The fluorescent antibody procedures are those described earlier by others, especially by Coons and his colleagues; a recent handbook (9) describes the general techniques in detail.

Complement fixation tests were done by the modified Kolmer procedure employed in this laboratory for diagnosis of viral and rickettsial diseases.

Results. 1. *Control of disruptive treatment.* That the mycobacteria were thoroughly opened up by the agitative treatment is shown in Fig. 1. Most of the protoplasm was released into the soluble fraction (B) (94% as judged by D_{265}^{PCA} determinations). This represented 70-80% of the dry weight of the intact organism. The sediment (disrupted bacilli, C) probably consisted chiefly of cell walls since its D_{265}^{PCA} value was only 9% of A, which would represent only 0.4 mg/ml. The spectrophotometer curves for the same 3 materials are shown in Fig. 2. The minimum for C at 240 was 87% of the maximum at 265, indicating that the D_{265}^{PCA} value overstates the protoplasmic content. It is not clear whether the protoplasm was present in this fraction as intact bacilli entrapped in the clumps, or as adhering protoplasm that did not leave the disrupted bacilli. As stated, dry weight values are imprecise at present because of inadequate bacterial mass.

Preparation of all the mycobacterial spe-

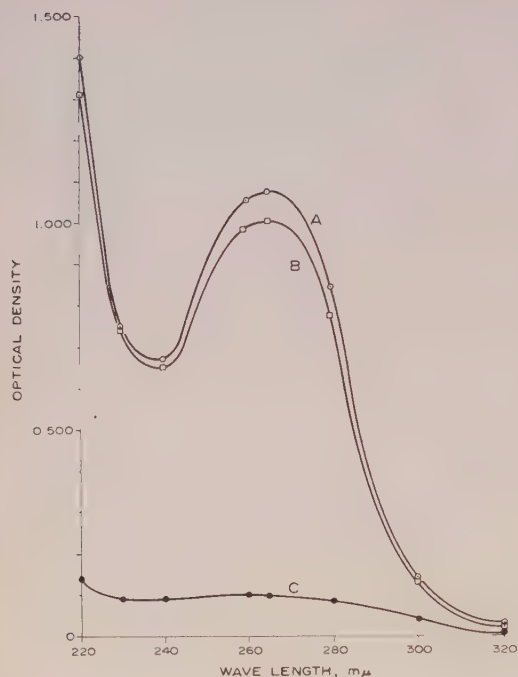


FIG. 2. Spectrophotometric curves of perchloric acid extracts of materials of Fig. 1. (A = intact bacilli, B = soluble fraction, C = disrupted bacilli.)

cies listed in Tables I and II have been monitored with bacterial counts, protein and D_{265}^{PCA} determinations. No important differences among species were noted. Incomplete break-up of the bacteria was revealed by low D_{265}^{PCA} for the supernate relative to the starting material and by lower protein values. The amplitude of vibration needs to be a full 7 mm to ensure efficient break-up in 5 minutes.

The disruption is associated with a variable amount of clumping of the remaining bacteria and cell walls, sometimes with attachment to glass surfaces. This leads to an overestimate of degree of bacterial disruption if judged by decrease in turbidity. It may also lead to poor recovery.

Acid-fast stains were not helpful in assessment of the efficiency of disruption. The smears were difficult to interpret quantitatively because of clumping and poor staining. There was a decrease in both acid-fast and nonacid-fast bacilli that roughly paralleled the degree of disruption indicated by

D_{265}^{PCA} and protein determination. Koch in 1897(10) described a loss of acid-fastness of tubercle bacilli when they were ground in the dried state in an agate mortar, and he used acid-fast stains to control the efficiency of grinding of tubercle bacilli during preparation of "TO" and "TR", which were the respective supernate and sediment of a subsequent centrifugation, and would be analogous to fractions B and C in Fig. 1. It seems likely that the process employed here releases more protoplasm into a soluble state, presumably because denaturation of protoplasmic constituents is avoided, and that loss of protoplasm results in loss of stainability with the methylene blue counterstain.

2. *Increase in stainability with fluorescent antibody that accompanies bacterial disruption.* Comparisons of stainability of several species of mycobacteria before and after disruption are given in Table I. No staining of bacterial bodies was observed with the first 5 mycobacteria, although there was some staining of the background between the organisms. Staining of the background is not very useful in fluorescent antibody studies because it lacks morphology and its specificity is difficult to control. The last 3 cultures, which are the rapidly growing species, could be well stained while still intact.

Addition of Tween 80 to a concentration of 0.05% or 0.5% (w/v) to the dilutions of antimycobacterial sera did not affect staining.

3. *Relative amounts of antigen available for antibody inhibition in the 3 fractions.* The antimycobacterial sera were diluted in the bacterial preparations to see if the staining could be inhibited. This approach is particularly convenient with fluorescent antibody methods because the indicating antigen is fixed to the slide and is not confused with the inhibiting (absorbing) antigen, which is carried away in the normal washing procedure(13). Representative examples of results for the various mycobacteria are given in Table II. The soluble fraction was the most effective inhibitor in each instance. The preparations of disrupted bacilli were not much more effective inhibitors than those of intact bacilli. The results of Table I sug-

TABLE I. Fluorescent Antibody Staining of Mycobacteria before and after Disruption by Vibration with Glass Beads. Indirect stain with antimycobacterial serum prepared in rabbits against the homologous culture. Numbers refer to brightness of staining (0 = no staining, 4 = brilliant staining) with antimycobacterial serum diluted 1:10.

Mycobacterial culture and abbreviation	Fluorescent antibody staining			
	Unbroken organisms (A)		Disrupted organisms (C)	
	Bacilli	Background	Bacilli	Background
Tb (<i>M. tuberculosis</i> , strain H37Rv)	0	0	4	4
BCG (Rosenthal vaccine strain)	0	2	3	3
Ka (<i>M. kansasii</i> (11), strain Barbee)	0	2	4	4
Ba ("Battey strain"(11), strain Wolfe)	0	2	3	3
NT and QT (hamster organism, Binford(12))	0	4	4	4
Fo (<i>M. fortuitum</i>)	3	3	3	3
Ph (<i>M. phlei</i>)	4	4	4	4
Sm (<i>M. smegmatis</i>)	3	3	4	4

gest that the inhibitory power of the latter 2 fractions reflects their content of the extra-bacillary antigen that produces background staining, rather than the amount of antigen located on the organisms or their cell walls and available to fluorescent antibody.

4. *Serological relationships between mycobacterial species.* A study of antigenic relationships among the mycobacterial species is being carried out with the rabbit sera and fluorescent antibody stains of the disrupted organisms. The results appear to follow the pattern described by Wilson(14). Cross-reacting antibody is usually present in high titer, and must be removed by absorption with the heterologous antigen to reveal specific homologous antibody. The serological differentiation of *M. tuberculosis* (strain H37Rv), and *M. kansasii* (strain Barbee) is illustrated in Table III.

5. *Considerations of specificity of staining of mycobacteria with fluorescent antibody.*

a) In low dilutions normal rabbit sera caused distinct staining of the broken-up organisms, but after immunization there was marked increase in titer. With direct procedures there was 2+ staining of some of the mycobacterial species studied with serum diluted 1:4, but after immunization there was always increase in staining, so that there was 4+ staining at the low dilutions and 2+ staining in dilutions of 1:16 or so. With the indirect procedure pre-immunization sera frequently gave 4+ staining in dilution of 1:10, and after immunization 4+ staining was observed with the rabbit sera diluted 1:100 to 1:1000. Staining of the mycobacteria was also observed with chicken and goat anti-rabbit conjugates in low dilutions, but it was not observed with the dilutions employed in the tests, i.e., 1:6 to 1:8. The lack of staining of the mycobacteria with the anti-rabbit conjugate only was controlled in each test. b) That the staining was not an

TABLE II. Relative Inhibitory Power of the 3 Types of Bacterial Preparations. Homologous antisera were diluted 1:10 in the 3 types of homologous antigen. Sera were then further diluted in VBA and applied to smears of disrupted bacilli (C), as the intermediate layer of an indirect fluorescent antibody stain. Symbols not defined in Table I are: Bl = *M. balnei* (strain X), and VBA = 2.5% bovine plasma albumin in veronal buffered saline.

Mycobacterium		VBA			Intact bacilli (A)			Soluble portion (B)			Disrupted bacilli (C)		
		10 ¹	10 ²	10 ³	10 ¹	10 ²	10 ³	10 ¹	10 ²	10 ³	10 ¹	10 ²	10 ³
1.	Tb	4	3	1	3	2	0	2	0	0	3	1	0
2.	Ka	4	3	1	3	2	0	0	0	0	2	1	0
3.	Ba	4	3	1	2	1	0	2	1	0	2	1	0
4.	NT	4	3	0	1	0	0	0	0	0	1	0	0
5.	QT	3	3	1	2	1	0	0	0	0	2	1	0
6.	Bl	4	3	2	3	3	0	2	0	0	4	2	0
7.	Fo	3	2	0	1	0	0	0	0	0	2	0	0

TABLE III. Serological Comparison of 2 Mycobacterial Species by Antibody Absorption. See Table I for definition of symbols.

Serum	Dil'n	VBA*		Tb*		Ka*	
		Tb†	Ka†	Tb	Ka	Tb	Ka
1. Anti-Tb	10 ¹	4	4	1	0	4	0
	10 ²	3	3	±	0	2	0
	10 ³	2	1	0	0	1	0
	10 ⁴	±	0	0	0	0	0
2. Anti-Ka	10 ¹	4	4	2	2	3	±
	10 ²	3	3	0	2	1	0
	10 ³	1	1	0	2	0	0
	10 ⁴	0	0	0	0	0	0

* Antisera were diluted in VBA, or in the soluble fraction (B) of Tb (*M. tuberculosis*), or of Ka (*M. kansasii*) before being used to stain mycobacterial antigens by indirect method.

† Smears were made from disrupted bacilli of *M. tuberculosis* or *M. kansasii*.

artefact produced by clumping of mycobacteria was obvious on comparison of stained and unstained smears, since staining gave the clumps a brilliant yellow-green color and rendered distinctly visible unclumped single bacilli, which were numerous in some preparations (Fig. 3 and 4). c) That the staining was due to fluorescent antibody and not some nonspecific fixation of labelled non-antibody protein, for example, by surface active forces, was attested by the increases in titer that occurred on immunization of rabbits, and by the specificity of the inhibition of staining that could be achieved by dilution of the serum in soluble preparations of antigen homologous to the mycobacterial species in the smear. d) The fluorescent antibody results, however, did not avoid the cross-reactions between mycobacterial species observed with other serological technics (*e.g.* 14). It seems probable that cross-reactions between mycobacterial species, and between mycobacteria and non-acid fast species, will make difficult the application of fluorescent antibody methods to laboratory diagnosis of mycobacterial diseases.

The soluble fractions (B) from *M. tuberculosis*, *M. kansasii*, *M. balnei*, and *M. phlei* were tested against the sera prepared against the same organisms in complement fixation tests. Block titrations were performed against homologous and heterologous sera. A condensation of the results with crosses among the 4 species is shown in Table IV.

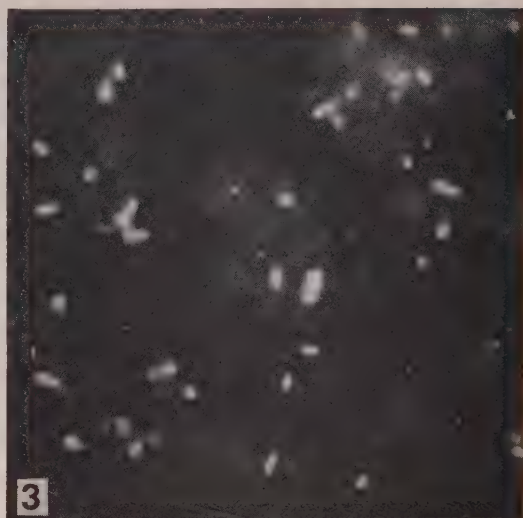


FIG. 3. Disrupted *Mycobacterium balnei* stained with fluorescent antibody. The bacilli, stained the typical yellow-green of fluorescein, were embedded in blue-gray autofluorescent amorphous material. $\times 885$.

FIG. 4. Control unstained smear of the same disrupted *M. balnei* preparation. Only the blue-gray autofluorescent amorphous material was visible. In preparations of intact organisms, staining was confined to background, and bacilli were not discernible. $\times 885$.

There was a helpful degree of specificity in each case.

6. *Antibody response to the 3 types of antigen preparations.* Only incomplete data are available. Rabbits immunized with each of the 3 preparations have responded satisfactorily with antibody, as shown by fluorescent antibody staining with an increase in

TABLE IV. Complement Fixation Titers of Rabbit Antisera Tested against Soluble Fraction (B) of Each of 4 Species, Tb (*M. tuberculosis*), Ka (*M. kansasii*), Bl (*M. balnei*), and Ph (*M. phlei*).

Serum	Antigen			
	Tb*	Ka*	Bl*	Ph*
Anti-Tb	>256†	64	64	16
" -Ka	64	>256	128	0
" -Bl	>256	32	>256	128
" -Ph	64	64	128	>256

* Antigen dilutions (2 units): Tb 80, Ka 320, Bl 320, Ph 80.

† Numbers refer to reciprocal of highest titer giving 3+ to 4+ fixation against 2 units of antigen.

titer of roughly 100-fold. The rabbits also showed strong antibody responses as demonstrated by agar diffusion and complement fixation. Studies of the efficacy of the preparations as vaccines are in progress.

Discussion. The intact mycobacteria of the more slowly growing species did not stain with fluorescent antibody and this is evidence that the outer surface does not contain antigen. This unusual condition presumably arises from the unique lipophilic surface of these organisms. When the rabbits were injected with the intact bacilli, they formed antibody that was not apparently different from that produced by injection of the soluble fraction or disrupted bacilli; probably the intact bacilli are broken up in the rabbit before it responds by formation of antibody.

Absorption by the soluble fraction of antibody capable of reacting with the disrupted bacilli might be interpreted to mean that there were no antigens present in the cell wall that were not also present in the protoplasm. There was not sufficient evidence of anatomical purity to establish such a conclusion, however, since protoplasm antigen might have persisted on the cell wall, or perhaps translocated there, and the soluble fractions (B) might have contained suspended fragments of cell wall.

Summary. 1) Mycobacteria were broken open by vibration with glass beads. Ninety to 95% of the protoplasm, and 70-80% of the bacterial mass was released into a soluble

state and could be separated by centrifugation from the sediment (disrupted bacilli), which consisted largely of cell walls. 2) Intact bacilli of the 5 more slowly growing species studied were not stainable with fluorescent antibody, but disrupted bacilli stained brightly. This was interpreted as evidence that the outer surfaces of intact bacilli of these species are free of antigen. 3) Intact bacilli of 3 of the more rapidly growing species stained well. 4) The soluble fraction contained the greatest amount of antigen as judged by ability to inhibit staining of the disrupted bacilli. It could be used to absorb (inhibit) cross-reacting antibodies to render immune sera more specific in their staining of disrupted organisms. 5) The soluble fraction was a potent antigen in agar diffusion tests. In complement fixation tests it was also an effective antigen with helpful specificity.

1. Mudd, E. B. H., Mudd, S., *J. Exp. Med.*, 1927, v46, 147.
2. Salton, M. R. J., in *The Bacteria. A Treatise on Structure and Function*. I. C. Gunsalus and R. Y. Stanier, Eds., Academic Press, New York, 1960, p97.
3. Ribi, E., Larson, C. L., List, R., Wicht, W., *Proc. Soc. Exp. Biol. and Med.*, 1958, v98, 263.
4. Ribi, E., Perrine, T., List, R., Brown, W., Goode, G., *ibid.*, 1959, v100, 647.
5. Shepard, C. C., *J. Exp. Med.*, 1960, v112, 445.
6. ———, *Am. J. Hyg.*, 1960, v71, 147.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.
8. Seifter, S., Dayton, S., Novic, B., Muntwyler, E., *Arch. Biochem.*, 1950, v25, 191.
9. Cherry, W. B., Goldman, M., Carski, T. R., *Fluorescent antibody techniques in the diagnosis of communicable diseases*. U. S. Govt. Printing Office, 1960.
10. Koch, R., *Deutsche Med. Wschr.*, 1897, v23, 209.
11. Runyon, E. H., *Med. Clin. N. Am.*, 1959, v43, 273.
12. Binford, C. H., *Lab. Invest.*, 1959, v8, 901.
13. Goldwasser, R. A., Shepard, C. C., *J. Immunol.*, 1959, v82, 373.
14. Wilson, G. S., *J. Path. Bact.*, 1925, v28, 69.

Received September 12, 1960. P.S.E.B.M., 1961, v106.

A Simple Device for Bedside Measurement of Carbon Dioxide Percentage in Expired Air. (26444)

FRANCES E. ROBERTS, MILTON B. COLE AND ALFRED H. LAWTON
Veterans Administration Center, Bay Pines, Fla.

One aspect of our long-term study of domiciliary members with emphysema has been the development of relatively simple bedside methods of sufficient accuracy for clinical determination of ventilatory function and expiratory gas exchange(1). Measurement of carbon dioxide content of alveolar air is very valuable but its value to the clinician decreases with the time required to procure a determination by the exacting micro Scholander technic. Three years ago we found a report of a simple device for measuring carbon dioxide content of flue gases which was said to be accurate within 2½%. On using the apparatus we found that the results obtained were sufficiently comparable for clinical purposes to those obtained by the more refined laboratory technics. The apparatus, known as the Fyrite CO₂ Indicator Model CND* consists of 2 conical chambers connected by a glass tube. The bottom chamber is filled with a 10% potassium hydroxide solution so that some fluid rises in the tube. The upper conical chamber has a valve through which the gas sample enters. An adjustable scale allows reading of the height

of the fluid before and after addition of the gas sample.

The apparatus measures CO₂ saturated with water. To check its accuracy using tank gas, which is dry, it is necessary to run the gas through water to saturate it. Respiratory gases, being water-saturated, need no such treatment.

Expired air may be collected in a bag and subsequently passed into the Fyrite, or preferably, the patient expires through a mouth-piece and tube. The last portion of the expired breath is discharged directly into the apparatus. No special valves are required. The entire operation requires no more than 5 minutes. The following chart shows comparative values of the same expired air gas samples in 20 normals, using the micro Scholander and the Fyrite methods.

The results obtained by the simple Fyrite method vary from those obtained by the micro Scholander technic by an average of .216. This is certainly accurate enough for clinical purposes.

Recently, Ravin and Stein(2) have described an instrument which seems to be ade-

TABLE I. Comparative Studies on Determination of CO₂ in Expired Air on Normal Control Group Using the Scholander Analyzer and Fyrite CO₂ Indicator - Model CND.

Subject	Scholander	Fyrite	Subject	Scholander	Fyrite
1	3.10	3.00	11	3.47	3.25
2	3.77	4.00	12	3.52	3.75
3	3.29	3.50	13	2.26	2.70
4	3.38	3.50	14	3.22	3.00
5	3.31	3.60	15	3.11	3.25
6	3.21	3.40	16	3.15	3.00
7	3.23	3.10	17	2.83	2.75
8	3.41	3.50	18	3.55	3.25
9	3.29	3.00	19	2.80	3.00
10	3.18	3.50	20	3.12	2.75

Avg difference .216

Scholander analyzer			Fyrite CO ₂ indicator - Model CND		
No. of cases	Mean and S.E. of mean		No. of cases	Mean and S.E. of mean	
20	3.20 ± .078		20	3.24 ± .055	

(p > 0.5)

* Bacharach Industrial Instrument Co., Pittsburgh, Pa.

quate for bedside studies. It requires much more care and time for its use than the apparatus described above.

We have also used the Fyrite Oxygen Indicator, which is similar in design and use to the CO₂ Indicator. In general, we have not found it as satisfactory when checked against the micro Scholander. This is of little importance since alveolar O₂ concentrations are of much less value to the clinician than the CO₂ concentration.

Summary. The Fyrite CO₂ Indicator Model CND is a satisfactory instrument for bedside measurement of carbon dioxide content of expired air.

1. Cole, M. B., Hammel, J. W., Manginelli, V. W., Lawton, A. H., Meindersma, M. S., *Dis. Chest*, 1960, v38, 519.

2. Ravin, H. A., Stein, M., *New Eng. Med. J.*, 1958, v259, 811.

Received October 5, 1960. P.S.E.B.M., 1961, v106.

Correlations Between Active Eyelid Closure and Depletion of Brain Biogenic Amines by Reserpine.* (26445)

E. COSTA AND G. R. PSCHIEDT

Thudichum Psychiatric Research Laboratory, Galesburg State Research Hospital, Galesburg, Ill.

In mice and rats active eyelid closure (blepharospasm) is used to evaluate the degree of sedation induced by reserpine(1). Neither guanethidine (15 mg/kg i.p.), which depletes norepinephrine stored in peripheral nerve endings(2), nor Bretyllium (20 mg/kg i.p.), which prevents its release(3), induces active eyelid closure in rats. This suggests that catecholamines stored at peripheral nerve endings are not directly involved in active eyelid closure induced by reserpine. The purpose of this paper is to elucidate the role played by depletion of brain biogenic amines in the mechanism of reserpine induced blepharospasm.

Our results suggest a positive correlation between active eyelid closure and depletion of serotonin (5HT) in the brain but fail to demonstrate a similar correlation with regard to brain norepinephrine.

Method. Male Wistar rats of 150 to 200 g were used in these experiments. Increase of motor output induced by drugs was evaluated with revolving cages. The animals were placed in the cage immediately after drug injection and kept there for 2 hours. A crossover design was used; the interval be-

tween retesting of each animal was one week. Eyelid closure was evaluated according to the method of Rubin *et al.*(1). Brain stem norepinephrine was determined according to Bertler *et al.*(4) using perchloric acid extracts. Brain stem 5 HT was measured by a bioassay technic using the rat uterus(5). Brain stems of 2 to 3 animals were combined and homogenized at 0°C with 19 volumes of 0.3 M ice cold sucrose containing 2.5×10^{-4} M of tranlylcyproamine. An aliquot (5cc) of the homogenate was removed for total 5 HT analysis, a second aliquot (10cc) was used for perchloric acid extraction and a third aliquot (5cc) of the homogenate was centrifuged in a Spinco Model L ultracentrifuge with rotor No. 40 for 40 min. at 20,000 rpm, equal to 26,360 g. Serotonin analysis was then performed separately in supernatant and precipitate. The monoamineoxidase inhibitor (MAOI) d-l tranlylcyproamine (SKF 385), Reserpine (Serpasil), and d-amphetamine sulfate were given alone or in combination to groups of at least 6 rats. Doses, route of injection, and time elapsed between treatments are indicated in Results.

Results. The effect of tranlylcyproamine on brain amines of rats is illustrated in Fig. 1. Two mg/kg i.p. of the drug caused an increase of brain 5 HT which reached its peak

* A preliminary report of these experiments was presented at Fed. Am. Soc. Exp. Biol., Chicago, April, 1960 (*Fed. Proc.*, 1960, v18, 279.).

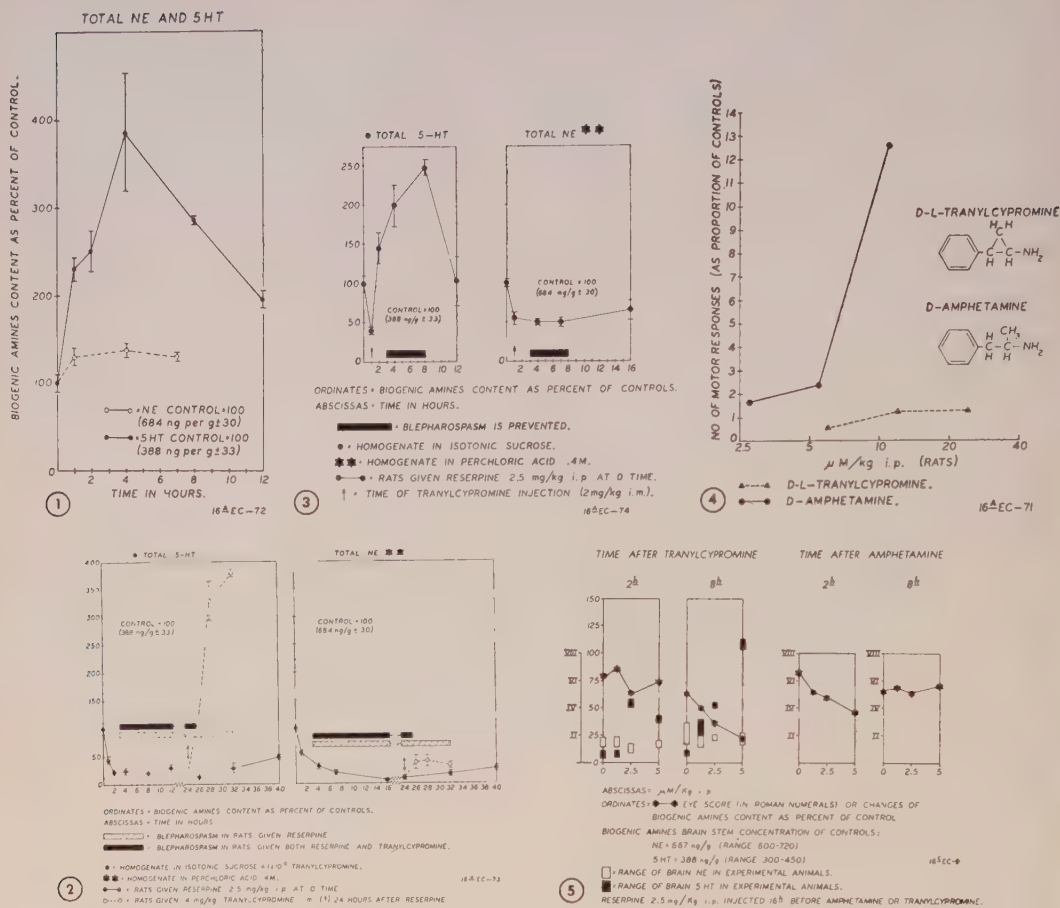


FIG. 1. Effect of d-l-tranlycypromine (2 mg/kg) on rat brain stem serotonin and norepinephrine concentration.

FIG. 2. Effect of reserpine alone and d-l-tranlycypromine 24 hr after reserpine on concentrations of rat brain stem amines.

FIG. 3. Effect of d-l-tranlycypromine given 1 hr after reserpine on rat brain stem amine concentrations.

FIG. 4. Effect of d-l-tranlycypromine and d-amphetamine on motor output of rats.

FIG. 5. Effect of various doses of d-l-tranlycypromine and amphetamine on reserpine-induced blepharospasm in rats.

within 4 to 6 hours and slowly declined toward control values during the next 6 hours. This dose of MAOI caused a small rise of brain norepinephrine. Higher doses of tranlycypromine induced a greater increase of catechol amines. The supernatant and precipitate of brain homogenates from rats given 2 mg/kg i.p. of tranlycypromine were analyzed separately for 5 HT. The supernatant accumulated 5 HT faster than the precipitate. The maximal increase of 5 HT was 3.7 and 2.5 fold respectively; however, the 2 fractions reached equilibrium within 8 hrs.

Reserpine (2.5 mg/kg) decreased the concentration of both 5 HT and norepinephrine in the brain stem of rats (Fig. 2). Depletion was greatest 12-26 hrs after injection, thereafter both amines increased towards control values. In these rats blepharospasm occurred 3 hrs after injection and endured for 29 hrs thereafter. Forty hrs after reserpine 5 HT levels were less than control but were equal to those present 3 hrs after reserpine when eyelid closure had not been established. In contrast, norepinephrine levels at this time equalled those found during blepharospasm.

Tranlycypromine (4 mg/kg) given 24 hrs after reserpine (Fig. 2) abolished the blepharospasm and concomitantly elevated the 5 HT concentration over control values. On the other hand, norepinephrine levels were only slightly elevated. Tranlycypromine (2 mg/kg) given 1 hr after reserpine reversed the active eyelid closure for a period of 5 hrs and the accompanying rise and fall of 5 HT in the brain paralleled the degree of reversal (Fig. 3). However, norepinephrine concentrations remained unchanged during period of reversal.

An attempt was made to determine whether the 5 HT which accumulated after administration of tranlycypromine to rats pretreated with reserpine was preferentially concentrated in either supernatant or precipitate. No such preferential distribution was found. Chemical changes in each fraction paralleled the changes in the whole homogenate.

Tranlycypromine and amphetamine are similar in chemical structure and both antagonized blepharospasm induced by reserpine. They differed considerably however in their action on motor activity in normal rats (Fig. 4) and in their time course of action in reserpinized rats (Fig. 5). Doses of tranlycypromine which produced extensive accumulation of 5 HT in the brain did not increase motor activity in rats when compared with equimolar doses of amphetamine. At all dose levels studied, tranlycypromine was more effective 8 hrs after injection in antagonizing reserpine-induced blepharospasm than at 2 hrs. The reverse was true for amphetamine.

Discussion. Tranlycypromine, an effective inhibitor of MAO(6), increased brain stem 5 HT levels markedly in both normal and reserpinized rats (Fig. 1, 2, 3). Norepinephrine levels were not elevated as much. Amphetamine and tranlycypromine both reversed reserpine-induced active eyelid closure. The antagonism displayed by tranlycypromine was related to its antienzymatic action rather than to an amphetamine-like central nervous system stimulation for the following reasons: (a) In normal animals 5 μ m/kg of tranlycypromine did not affect motor

output when compared with 5 μ m/kg of amphetamine (Fig. 4). (b) This dose of tranlycypromine was still effective on eyelid closure 8 hrs after injection whereas amphetamine was maximally effective 2 hrs after injection and was not effective 6 hrs thereafter (Fig. 5). (c) The antagonism produced by MAOI was paralleled by an increase of brain stem 5 HT. (d) It is unlikely that amphetamine reverses eyelid closure by restoring the deficit of brain norepinephrine since the MAOI reversed reserpine effects without markedly changing norepinephrine concentrations.

These results therefore support Brodie's hypothesis(7) that release of brain 5 HT rather than norepinephrine is important for understanding the mechanism of action of reserpine. Other amines such as dopamine may also be important. These findings do not clarify the mode of action of reserpine on 5 HT binding sites. It may be suggested that adverse effects of reserpine on 5 HT transport mechanisms(8) can be overcome only when appropriate concentrations of free 5 HT are accumulated intracellularly as a result of MAO inhibition. For example, when 5 HT concentrations in the brain stem were brought back to control levels (Fig. 2, 3) reserpine-induced blepharospasm was abolished. It is not clear why tranlycypromine had less effect on brain norepinephrine than on brain 5 HT. This finding could result from either slower turnover of norepinephrine or lack of action of tranlycypromine on other enzyme systems involved in the metabolism of norepinephrine in brain tissue (9).

Summary. Reserpine-induced closure of the eyelids is antagonized by amphetamine and tranlycypromine *via* different mechanisms. Antagonism by tranlycypromine is a function of increased 5 HT content in the brain stem but is independent of changes in norepinephrine concentration.

1. Rubin, B., Malone, M. H., Waugh, M. H., Burke, J. C., *J. Pharm. Exp. Therap.*, 1957, v120, 125.
2. Cass, R., Kuntzman, R., Brodie, B. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v103, 871.
3. Boura, A. L. A., Geen, A. F., *Brit. J. Pharmacol. Chemotherapy*, 1959, v14, 536.

4. Bertler, A., Carlsson, A., Rosengren, E., *Acta Physiol. Scand.*, 1958, v44, 273.
5. Garven, J. D., *Brit. J. Pharmacol. Chemotherap.*, 1956, v11, 66.
6. Horita, A., McGrath, W. F., *Biochem. Pharmacol.*, 1960, v3, 206.
7. Brodie, B. B., Finger, K. F., Orlans, F. B., Quinn, G. P., Sulser, F., *J. Pharmacol. Exp. Therap.*, 1960, v129, 250.
8. Hughes, F. B., Brodie, B. B., *ibid.*, 1959, v127, 96.
9. Axelrod, J., *Science*, 1958, v127, 759.

Received November 9, 1960. P.S.E.B.M., 1961, v106.

Effect of Drugs on Amino Acid Levels in Brain: Excitants and Depressants. (26446)

R. S. DE ROPP AND E. H. SNEDEKER (Introduced by B. L. Hutchings)

Biochemistry Research Dept., Biochemical Research Section, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Previous studies on the action of drugs on amino acid levels in the brain have brought to light a variety of effects. Okumura, *et al.* (1), using ion exchange chromatography to analyze brain amino acids, found that repeated injections of chlorpromazine produced a rise in glutamine, aspartic acid and γ -aminobutyric acid (GABA). Repeated electroshock treatment and repeated injections of β -phenylisopropylmethylamine produced no marked changes in free amino acid levels, although the latter produced an increase in N-acetylaspartic acid. That a reduction occurs in the level of GABA in brains of rats treated with semicarbazide was demonstrated by Killam and Bain(2), but this effect was not observed in animals with seizures induced by pentylenetetrazole. Baxter and Roberts (3) found significant increases in GABA levels in brains of rats treated with hydroxylamine.

The effect of 4 hypoglycemic agents on free amino acid levels in the rat brain has been described(4). The present paper describes results obtained by applying the same analytical procedures(5) to a study of a larger group of agents affecting the central nervous system. These were divided into compounds exerting a predominantly stimulating effect (including the so-called hallucinogens, mescaline and LSD) and compounds exerting a predominantly depressing effect (hypnotics, analgesics and "tranquilizing" agents).

Experimental. A. *Design of experiments.* Wistar rats (250 g males) were fasted 24

hours, then injected with the material to be tested dissolved in normal saline. Amounts injected varied and have been recorded under Results. The amount of time allowed for the drug to act also varied. In every experiment 6 groups of 5 rats were used, the experiment being so designed that 2 groups functioned as controls (injected with normal saline), 2 groups received one compound to be tested, and the remaining 2 groups received the second compound to be tested. Thus each experiment consisted of 30 rats distributed as shown below:

Group	Control	Compound A	Compound B	Total
I	5	5	5	15
II	5	5	5	15
	10	10	10	30

Each of these unit experiments was repeated 2 or more times, the brains being extracted in one experiment with picric acid and in the other with alcohol. Both extraction procedures and chromatographic methods of determining amino acid levels have been described(5).

Estimations of the significance of treatment effects were made by the method of paired replicates as described by Snedecor (6). This involved obtaining the difference in density reading between each of the treated groups and its own control in each experiment. From the sum of such differences derived from all the experiments carried out, a mean difference was obtained, the

TABLE I. Free Amino Acid Levels (As % of Controls) in Brains of Rats Treated with Agents Having Predominantly Stimulating Effects. (No. of estimates in parentheses.)

Treatment	Dose, mg/kg	Amino acid									
		GSH	Asp.	Glu.	Gln. NH ₂	Gly. Ser.	GABA	Ala.	Tau.	Etolh. amine	P. eth. amine
Pentylenetetrazole	100	102 (6)	93 (9)	87 (10)	113 (6)	109 (2)	107 (14)	184 (10)	102 (5)	145 (4)	119 (2)
Semicarbazide	500	103 (11)	81 (4)	97 (9)	—	—	62 (13)	119 (13)	—	—	92 (6)
Strychnine	4	130 (4)	—	110 (4)	—	—	115 (4)	166 (4)	—	—	—
Picrotoxin	12	102 (8)	98 (10)	106 (12)	—	—	105 (9)	151 (9)	112 (1)	—	141 (6)
Electroshock (convulsion)	—	104 (8)	95 (6)	109 (10)	105 (2)	103 (2)	109 (10)	128 (10)	109 (2)	—	119 (2)
Electroshock (recovery)	—	120 (8)	104 (6)	110 (10)	115 (2)	168 (2)	106 (10)	131 (10)	106 (2)	—	128 (1)
Caffeine	240	91 (4)	94 (6)	102 (8)	105 (2)	—	104 (4)	115 (4)	100 (2)	—	—
Pheniprazine	80	88 (8)	103 (4)	103 (10)	106 (3)	—	106 (8)	130 (8)	117 (8)	117 (8)	—
Amphetamine	100	110 (20)	96 (12)	107 (22)	108 (5)	—	83 (18)	148 (16)	100 (6)	—	—
Mescaline	240	86 (12)	97 (4)	106 (12)	108 (2)	—	108 (8)	130 (8)	90 (2)	—	—
LSD	200 μ g	102 (8)	110 (10)	101 (10)	105 (2)	—	107 (10)	93 (10)	100 (6)	—	—

standard error of which was used to obtain a value for *t* and a corresponding value for *P*. Increases or decreases in amino acid levels have been expressed as percentages of the relevant controls and the significance of the changes, calculated as described above, has been recorded. Following accepted usage values of *P* of .001 or less have been regarded as highly significant, between .01 and .001 as significant and between .05 and .01 as possibly significant.

Results. A. Stimulants. The effects on brain amino acid levels of 10 agents exerting a predominantly stimulating effect on the central nervous system are shown in Table I. The following clinical effects and significant changes in amino acid levels were observed.

Pentylenetetrazole (100 mg/kg) produced severe convulsions. The rats were killed 30 minutes after injection when these convulsions were at their height. Significant changes in amino acid levels (expressed as percentages of controls) were glutamic, 87% ($P < .01$); glutamine, 113% ($P < .02$); alanine, 184% ($P < .001$). A rise in ethanolamine (145%), though large, was of questionable significance.

Semicarbazide (500 mg/kg) produced convulsions 45 to 60 minutes after treatment. Rats were killed during these convulsions. Significant changes in amino acid levels were GABA, 62% ($P < .001$) and alanine, 119% ($P < .01$).

Strychnine (4 mg/kg) produced severe convulsions 10 minutes after injection. The rats were killed during these convulsions. A significant rise took place in the level of alanine (166%, $P < .01$). A rise in GABA (115%) was of questionable significance.

Picrotoxin (12 mg/kg) produced convulsions during which the animals were killed. The rise in level of alanine was highly significant (151%, $P < .001$). The rise in phosphoethanolamine (141%) was not consistent enough to be significant.

Electroshock (150 mA) was applied directly to the cornea for 0.3 seconds. Rats went into convulsions followed by coma. Half of the rats were killed during the convulsions (about 30 seconds after shock), the other half as they were recovering from the

TABLE II. Free Amino Acids (As % of Controls) in Brains of Rats Treated with Agents Having Predominantly Depressant Effects. (No. of estimates in parentheses.)

Treatment	Dose, mg/kg	Amino acid							Eroh. amine
		GSH	Asp.	Glu.	Glu. NH ₂	Gly. Ser.	GABA	Ala.	
Meprobamate	260	103 (12)	109 (10)	108 (10)	93 (2)	—	108 (8)	92 (8)	75 (2)
Chlorpromazine	100	98 (13)	104 (6)	94 (13)	141 (8)	—	105 (13)	118 (13)	99 (4)
Methoxypropazine	200	104 (4)	96 (2)	97 (6)	120 (2)	—	111 (4)	130 (4)	105 (2)
Reserpine	50	91 (13)	94 (10)	101 (15)	103 (2)	—	108 (15)	100 (15)	88 (6)
Phenobarbital	120	101 (12)	93 (5)	86 (15)	100 (4)	93 (4)	88 (13)	97 (10)	85 (3)
Chloral hydrate	240	104 (4)	103 (2)	98 (4)	87 (2)	—	95 (4)	91 (4)	92 (2)
Diphenylhydantoin	400	98 (7)	104 (9)	101 (14)	102 (8)	105 (6)	101 (13)	113 (9)	97 (8)
Acetazolamide	500	92 (4)	99 (6)	84 (8)	103 (2)	—	82 (8)	97 (6)	98 (2)
Morphine	240	87 (4)	76 (4)	71 (4)	—	—	94 (4)	114 (4)	—
	10	—	108 (8)	108 (16)	102 (8)	91 (8)	110 (16)	105 (16)	105 (8)

coma (about 15 minutes after shock). In the brains of rats killed during convulsions there was a rise in alanine (128%, $P < .02$). In the brains of rats killed during the recovery stage this rise was still present (alanine, 131%). The rises in phosphoethanolamine observed were of doubtful significance.

Caffeine (240 mg/kg) did not produce any obvious excitation in the rats which were killed $1\frac{1}{2}$ hours after injection. A rise in alanine (115%) was observed but its significance was doubtful. Pheniprazine (80 mg/kg) produced excitement in the rats which became extremely vicious. They were killed $2\frac{1}{2}$ hours after injection. A rise in level of alanine to 130% of controls was highly significant ($P .001$). A rise in level of ethanolamine (117%) was of questionable significance. Amphetamine (100 mg/kg) produced extreme excitement in the rats which were killed while in the excited state. A rise in alanine to 148% of control value was highly significant ($P < .001$). A fall in GABA to 83% was apparently significant ($P .01$) but considerable variation occurred in the magnitude of this effect in different experiments.

The effects of 2 hallucinogenic drugs on brain amino acid levels were compared. Mescaline (240 mg/kg) produced extreme excitement in the rats which fought so viciously that they had to be separated. They were killed while in the excited state. A rise in alanine (130%) was significant ($P < .01$). A fall in glutathione (86%) was of doubtful significance. Lysergic acid diethylamide (200 μ g/kg) did not have an outwardly observable effect on the rats. They were killed $2\frac{1}{2}$ hours after injection. No significant changes in level of their amino acids could be detected.

B. Depressants. The effects of 2 hypnotic agents on brain amino acid levels were compared. Phenobarbital (120 mg/kg) reduced the rats to a state of somnolence in which condition they were killed $3\frac{1}{2}$ to 4 hours after injection. A fall in glutamic acid to 86% of controls was significant ($P < .01$) and a fall in level of GABA to 88% was also significant ($P < .01$). Chloral hydrate (240 mg/kg) did not produce observable effects on the rats which were killed $3\frac{1}{2}$ to 4 hours

after injection. No significant changes in brain amino acid levels could be detected in animals treated with this drug.

Acetazolamide (500 mg/kg) did not produce observable physiological effects but lowered the level of brain glutamic acid to 84% of control value ($P < .01$) and lowered GABA level to 82% (P nearly .02). Diphenylhydantoin (400 mg/kg) also produced no observable effect and did not significantly alter levels of brain amino acids.

The effects of 4 tranquilizing agents were compared. Meprobamate (260 mg/kg) produced symptoms of ataxia in the rats which were sacrificed $3\frac{1}{2}$ hours after injection of the drug. No significant changes in levels of amino acids could be detected in the brains of these animals. Chlorpromazine (100 mg/kg) induced a semi-comatose condition in the rats which were sacrificed 4 hours after injection. A rise in glutamine to 141% of control level was highly significant ($P < .001$). A rise in alanine to 118% was also significant (P nearly .001). Methoxypromazine (200 mg/kg) produced drowsiness in the animals which were sacrificed 4 hours after injection. Like chlorpromazine this drug produced a rise in level of brain glutamine (120%) which was significant. It also produced a significant rise in alanine (130%, $P < .01$). Reserpine was administered at the rate of 50 mg/kg per day for 3 days. The animals remained asleep for the last 24 hours and were sacrificed while in that state. No significant changes in brain amino acid levels were observed.

One analgesic, morphine, was tested at 2 dose levels, 240 and 10 mg/kg. At the very high level this drug produced a fall in aspartic acid to 76% of the controls and of glutamic acid to 71%. At the lower dose it did not produce significant changes in brain amino acid levels.

Discussion. This study was designed to determine whether agents having in common a certain type of physiological action would also have certain common effects on the metabolism of the brain as reflected in the level of free amino acids in this organ. The 4 convulsant agents tested had one property in common with electroshock. All of them

brought about a rise in level of brain alanine ranging from 19% increase produced by semicarbazide to 84% increase produced by pentylenetetrazole. Semicarbazide has already been shown by Killam and Bain(2) to produce a fall in level of GABA in the brain, a finding which is confirmed by the present work. Such a fall in GABA was not produced by the other convulsants tested. It is impossible, for this reason, to attribute the convulsant action of all these agents to a lowering of the level of GABA. Several of these drugs produced rises in level of ethanolamine but because of the low level of this compound in the brain the significance of the rise was questionable.

The excitant drugs, pheniprazine and amphetamine, shared with the convulsant agents the property of increasing brain alanine levels. Caffeine, a milder excitant, did not have this capacity. The hallucinogen, mescaline, produced excitation in the rats and also produced the rise in brain alanine characteristic of excitants. Lysergic acid diethylamide did not produce excitation and had no effect on alanine levels.

The hypnotic agent, phenobarbital, produced a fall in level of both glutamic acid and GABA in the brain, but the chemically unrelated hypnotic, chloral hydrate, did not have this effect. The anti-convulsant, acetazolamide, resembled phenobarbital in producing a fall in level of glutamic acid and GABA, but a second anti-convulsant, diphenylhydantoin, did not have this effect. According to Woodbury and Vernadkis(7) injections of diphenylhydantoin will raise the level of GABA in the rat brain provided the animals have been adrenalectomized. Baxter and Roberts(3) found that "only slight and inconsistent elevations in the GABA levels" were produced in the brains of rats injected with acetazolamide. It does not appear that these anti-convulsants can exert their action by raising GABA levels.

The 2 phenothiazine tranquilizers, chlorpromazine and methoxypromazine both brought about a rise in level of brain glutamine. Okumura *et al.*(1) using chlorpromazine also detected such a rise. A rise in brain alanine was also produced by these 2 sub-

stances. Neither reserpine nor meprobamate produced significant change in level of brain amino acids. The analgesic, morphine, though it produced a lowering in aspartic and glutamic acid levels when an excessively high dose was used, did not exert any effect at a dose level which was still quite adequate to produce analgesia.

Thus, the only correlation between pharmacological activity and biochemical action which emerges from this work is that between the rise in brain alanine and convulsant or excitant activity. Even this is not clear cut because the two phenothiazine tranquilizers tested both produced a rise in alanine levels in the brain although they are not excitants. The rise in glutamine produced by chlorpromazine and methoxypropazine may be characteristic of phenothiazines in general but can hardly be correlated with tranquilizing activity as neither meprobamate nor reserpine exerted this effect.

Summary. 1. Effect of electroshock and 18 chemical agents (pentylentetrazole, semicarbazide, strychnine, picrotoxin, caffeine, pheniprazine, amphetamine, mescaline, LSD, phenobarbital, chloral hydrate, acetazolamide, diphenylhydantoin, meprobamate, chlorpromazine, methoxypropazine, reserpine and morphine) was determined on the level of 10 ninhydrin-reacting compounds in

the rat brain (glutathione, phosphoethanolamine, aspartic acid, glutamic acid, GABA, glutamine, glycine + serine, alanine, taurine and ethanolamine). 2. The convulsant agents, pentylentetrazole, semicarbazide, strychnine, picrotoxin and electroshock as well as the excitants, pheniprazine, amphetamine and mescaline, brought about significant rises in free alanine in the brain. Lowering of the GABA levels to 60% of that of the controls was brought about by semicarbazide but not by any of the other convulsant agents. 3. Two phenothiazine tranquilizers chlorpromazine and methoxypropazine, brought about a significant rise in level of brain glutamine.

1. Okumura, N., Otsuki, S., Nasu, H., *J. Biochem.* (Tokyo) 1959, v46, 247.

2. Killam, K. F., Bain, J. A., *J. Pharm. Exp. Therap.*, 1957, v119, 255.

3. Baxter, C. F., Roberts, E., *The Neurochemistry of Nucleotides and Amino Acids*, R. O. Brady and D. B. Tower, Ed., Wiley and Sons, N. Y., 1960.

4. De Ropp, R. S., Snedeker, E. H., *J. Neurochem.*, in press.

5. ———, *Anal. Biochem.*, 1960, v1, 424.

6. Snedecor, D. W., *Statistical Methods*, 1956, 5th ed., Iowa State College Press, Ames.

7. Woodbury, D. M., Vernadakis, A., *Fed. Proc.*, 1958, v17, 420.

Received November 21, 1960. P.S.E.B.M., 1961, v106.

Effects of Quinidine on Triacetin Utilizing Activity of Pancreas.* (26447)

GERALD LITWACK AND PEARLEE SHRAGER

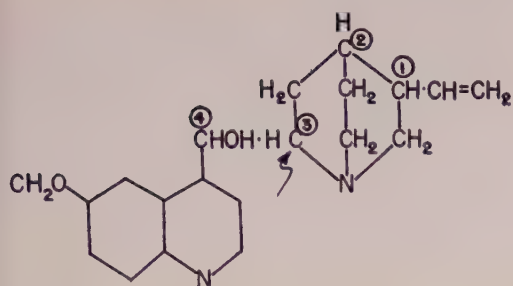
Biochemical Laboratory, Division of Cardiology, Philadelphia General Hospital and Graduate School of Medicine, University of Pennsylvania, Philadelphia

Rona and Reinicke(1) demonstrated that serum lipase, using tributyrin as substrate, was inhibited by quinine such that the logarithm of the inhibitor concentration plotted against the velocity of the reaction produced a straight line. A protective effect of tributyrin was not observed. Later, Hellerman *et al.*(2) mentioned that both quinine and ata-

brine inhibited pancreatic lipase reversibly, although no data were presented.

No reports have been seen in the literature which describe the action of quinidine upon triacetin utilizing activity (TUA). Quinidine, a stereoisomer of quinine, appears to differ from quinine only in the spatial arrangement of the asymmetric carbon 3, the arrangements of the asymmetric centers, carbons 1, 2 and 4 apparently being identical (3),

* This study was supported by a grant from Southeastern Heart Assn. and Pennsylvania Heart Assn.



Quinidine is dextrorotatory compared to the levorotatory character of quinine. Quinidine is of special importance in treatment of cardiac arrhythmias and is administered in levels which feasibly could result in inhibition of intestinal triglyceride breakdown, presumably a beneficial side effect in cardiac patients.

Quinine is generally known to act as a competitive inhibitor of flavineadeninedinucleotide coenzymes in a fashion similar to quinolines and auramines(2).

The present paper reports upon the nature of the interaction of quinidine and pancreatic TUA. Some of the features of this interaction have been reproduced with wheat germ lipase.

Experimental. A dried hog pancreatic preparation was obtained commercially from Nutritional Biochemicals Corp. (Cleveland, Ohio) and wheat germ lipase, prepared according to the specifications of Singer(4), was obtained from the same commercial source. **TUA Assay.** Essentially, the manometric method of Singer and Hofstee(5) for lipase was used. A nitrogen atmosphere was used throughout the studies. Linearity of enzyme concentration and carbon dioxide production occurred over a given range and all studies were conducted within these limits. Data obtained with wheat germ lipase also reflected enzyme levels which produced a linear relationship to carbon dioxide evolution. Triacetin was used as the substrate in all cases and was added from the side arm after 10 minutes incubation at 37°C under nitrogen unless otherwise indicated. Readings were made every 10 minutes for one hour or longer.

Results and discussion. The effect of increasing concentrations of quinidine sulfate

upon TUA is shown by Fig. 1. A marked inhibition of TUA is not observed until the level of quinidine sulfate is greater than 10^{-3} M. However, some depression of TUA activity (Fig. 1) occurred in the region of 10^{-5} M which appeared to be statistically significant. The level of 5.10^{-3} M quinidine was therefore selected to determine the kinetic behavior of this interaction.

The results of a typical Ackermann-Potter type inhibition curve(6) suggested a reversible inhibition. This was confirmed using double reciprocal plots of the Lineweaver-Burk type(7). Since the inhibition was thought to be of the reversible type, an experiment was designed to test the possible reversal of quinidine sulfate inhibition by substrate. A typical result of such a study, shown in Fig. 2, shows the possibility for at least partial reversal of inhibition by substrate addition. Curve D has a greater slope than the reversal curve B, which indicates that the reversal is incomplete and may be calculated to show that the reversal under these conditions amounts to about 70%.

Because the inhibition was reversible by substrate an experiment was designed to test the possibility of substrate protection. A typical result is demonstrated in Fig. 3. Little or no substrate protection is afforded under these conditions, and curiously enough, a similar result was obtained by Rona and Reinicke(1) who used human serum lipase, tributyrin substrate, and quinine as the inhibitor.

Fig. 4 illustrates the effect of heparin upon TUA activity in presence or absence of quinidine sulfate. Ten mg heparin per vessel produced about 30% inhibition of TUA activity and in the presence of quinidine, inhibition is increased by heparin by about 20%. This suggests that heparin and quinidine may operate at different loci. Inhibition of TUA with Tween 60 as substrate, by 5.10^{-4} M heparin has been reported by Katz(8).

The effects of various metals upon TUA activity are represented in Table I. Fe^{+3} is practically without effect and does not influence quinidine inhibition. On the other hand, both Cu^{+2} and Mg^{+2} are inhibitory. The presence of Cu^{+2} ion slightly increases

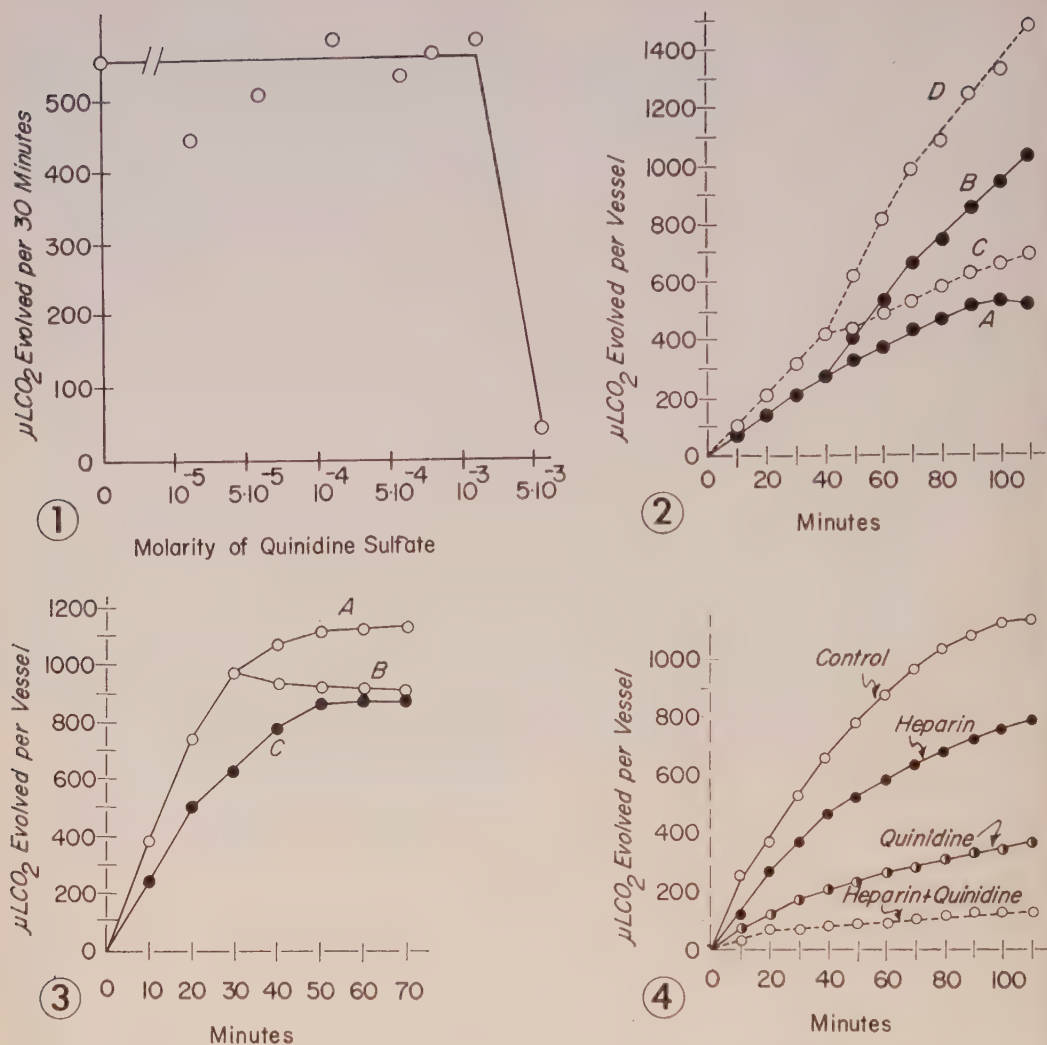


FIG. 1. Effect of quinidine sulfate on TUA activity. Illustrates one of 21 similar experiments.

FIG. 2. Partial reversal of quinidine sulfate inhibition of TUA activity by triacetin substrate. Curve A, $5 \cdot 10^{-3}$ M quinidine sulfate in suspension was incubated with the test system using 265 μmoles triacetin in the standard system; Curve B, same system as in A except that 530 μmoles triacetin are added after 40 min. of incubation. Curve C, standard system incubated with 265 μmoles triacetin in absence of quinidine sulfate. Curve D, same system as in C except that 530 μmoles triacetin are added after 40 min. of incubation. Illustrates one of 6 similar experiments.

FIG. 3. Attempt to protect from quinidine inhibition by substrate. Curve A, standard system with 795 μmoles triacetin. Curve B, same system as in A except that $5 \cdot 10^{-3}$ M quinidine sulfate is added after 30 min. incubation. Curve C, standard system as in A except that $5 \cdot 10^{-3}$ M quinidine sulfate is added at time zero. Illustrates one of 5 similar experiments.

FIG. 4. Effects of heparin upon TUA activity in presence or absence of $5 \cdot 10^{-3}$ M quinidine sulfate. Ten mg heparin was used per system. Illustrates one of 6 similar experiments.

the inhibition by quinidine, however, Mg^{+2} ions do not appear to elicit a similar response. It is assumed that the metals are probably not forming significant quantities of soaps with the released fatty acids, in which case

it would be expected that the reaction would be stimulated by a Mass Action effect. The possibility, however, cannot be completely ruled out. These studies were extended to determine what effect, if any, ethylenedia-

TABLE I. Effects of Metals upon Pancreatic TUA in Presence or Absence of Quinidine.

Exp.	No. of exp.	TUA activity (μ l CO ₂ /30 min.)	% of control exp.
Control	6	556 \pm 26†	100
" + quinidine sulfate*	6	178 \pm 5	32
" + FeCl ₃	3	500 \pm 25	90
" + quinidine sulfate + FeCl ₃	3	175 \pm 11	32
" + CuCl ₂	6	175 \pm 6	32
" + quinidine sulfate + CuCl ₂	6	102 \pm 9	18
" + MgCl ₂	3	420 \pm 56	76
" + quinidine sulfate + MgCl ₂	3	167 \pm 8	30

* Concentrations used: Quinidine sulfate, 5.10^{-3} M suspension; FeCl₃, or CuCl₂, or MgCl₂, 10^{-3} M; all solutions adjusted to pH 7.0 before use.

† \pm stand. error of mean.

minetetraacetic acid (EDTA) would have upon TUA inhibition by Cu⁺². The results are presented in Table II. Cu⁺² again enhances inhibition by quinidine or vice versa. EDTA itself inhibits TUA activity, which suggests that a metal component may occur as an activator or stabilizer of TUA or that EDTA may prevent an hypothetical salt formation of the released free fatty acids which could produce an inhibition. The former hypothesis has received considerable support (9,10) and Wills(10) has recently confirmed

TABLE II. Effect of EDTA on Pancreatic TUA and Its Inhibition by Cu⁺² and Its Stimulation by Ca⁺² in Presence or Absence of Quinidine.

Exp.	No. of exp.	Relative TUA activity (μ l CO ₂ /30 min.), % of control
Control	4	100
" + quinidine SO ₄ †	4	27 (23-31)*
" + Cu acetate	4	28 (24-37)
" + quinidine SO ₄ + Cu acetate	4	15 (10-18)
" + EDTA	4	31 (27-40)
" + quinidine SO ₄ + EDTA	4	21 (14-41)
" + Cu acetate + EDTA	4	52 (39-64)
" + quinidine SO ₄ + Cu acetate + EDTA	4	24 (13-40)
" + CaCl ₂	4	145 (125-160)
" + CaCl ₂ + EDTA	4	54 (49-67)
" + quinidine SO ₄ + CaCl ₂	4	34 (30-41)
" + quinidine SO ₄ + CaCl ₂ + EDTA	4	7 (4-12)

* Range.

† Concentrations used: Quinidine SO₄, 5.10^{-3} M in suspension; cupric acetate, 10^{-3} M; EDTA, 10^{-3} M; CaCl₂, 10^{-3} M; all solutions adjusted to pH 7.0 before use.

that Ca⁺² acts as a stabilizing metal for lipase. The data shown in Table II indicate a stimulation of TUA activity by Ca⁺² and more specifically that inhibition of TUA activity by EDTA is in part reversed by Ca⁺² ions. TUA is only slightly stimulated even in the presence of quinidine which may suggest that the action of quinidine may not involve the Ca⁺² ion site on the enzyme.

Comparisons of kinetic studies show that quinidine gluconate is 2 to 3 times more effective as an inhibitor of TUA than quinidine sulfate, possibly because of its greater solubility. Quinine sulfate is shown by the inhibitor constant to be more effective than quinidine. A similar study was carried out with wheat germ lipase (WGL) and the apparent relationship between quinidine and quinine is also reflected in these experiments (Table III). The Michaelis-Menten constant for WGL is about one-half that for TUA using triacetin as substrate. Because triacetin is a low-molecular weight triglyceride it cannot be stated that the results with the pancreatic preparation are exclusively due to pancreatic lipase; on the other hand, many of the results have been reproduced with wheat germ lipase.

Summary. Pancreatic TUA is reversibly inhibited by quinidine and quinine. Quinidine inhibition can be reversed by triacetin to about 70%; however, protection by triacetin could not be demonstrated. Quinidine and related compounds are effective as inhibitors of pancreatic triacetin utilizing activity in the following order: quinine sulfate > quinidine sulfate; quinidine gluconate >

TABLE III. *In Vitro* Inhibition of Wheat Germ Lipase by Suspensions of Quinidine Sulfate or Quinine Sulfate.*

Inhibitor	No. of exp.	K_s (moles triacetin/l) \pm SEM	K_i (\pm SEM)
Quinidine sulfate	5	$2.3 \pm .2 \times 10^{-1}$	$1.0 \pm .5 \times 10^{-3}$
Quinine sulfate	3	$1.9 \pm .3 \times 10^{-1}$	$5.7 \pm .5 \times 10^{-4}$

* Performed at 37°C under N₂ with a 10 min. preincubation.

quinidine sulfate. To the extent tested, the same relationship holds for wheat germ lipase using triacetin as substrate. In the wheat germ lipase system the K_i for quinidine sulfate = 1×10^{-3} , and the K_i for quinine sulfate = 5.7×10^{-4} . Heparin inhibits the pancreatic TUA in presence or absence of quinidine. Metals, such as Cu⁺² and Mg⁺² inhibit this system and Cu⁺² increases quinidine inhibition but Mg⁺² does not. Fe⁺³ does not inhibit the system or interfere with quinidine inhibition. EDTA inhibits this system by 70% at a level of 10^{-3} M. This inhibition is partially reversed by Ca⁺² ions which also markedly stimulate TUA activity. EDTA does not greatly influence quinidine inhibition. Cu⁺² inhibition is partially reversed by EDTA in spite of the fact that EDTA is an inhibitor. EDTA reduces the stimulatory effect of Ca⁺². Quinidine inhibition is only slightly lessened by

Ca⁺² so that it appears that the site of action of quinidine may not involve a Ca⁺² ion stabilizer.

1. Rona, P., Reinicke, D., *Biochem. Z.*, 1921, v118, 213.
2. Hellerman, L., Lindsay, A., Bovarnick, M. R., *J. Biol. Chem.*, 1949, v163, 553.
3. Karrer, P., *Organic Chemistry*, Elsevier Publ. Co., Inc., 4th Ed., New York, 1950, 874.
4. Singer, T. P., *J. Biol. Chem.*, 1948, v174, 11.
5. Singer, T. P., Hofstee, B. H. J., *Arch. Biochem.*, 1948, v18, 229.
6. Ackermann, W. W., Potter, V. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, v72, 1.
7. Lineweaver, H., Burk, D., *J. Am. Chem. Soc.*, 1934, v56, 658.
8. Katz, S., *Am. J. Gastroenterol.*, 1957, v27, 479.
9. Willstatter, R., Memmen, F., *Z. Physiol. Chem. Hoppe Seyler's*, 1929, v133, 229.
10. Wills, E. D., *Biochim. et Biophys. Acta*, 1960, v40, 481.

Received December 6, 1960. P.S.E.B.M., 1961, v106.

Oxidation of Ergosterol by Rat and Mouse Liver Mitochondria.*† (26448)

DAVID KRITCHEVSKY, EZRA STAPLE† AND MICHAEL W. WHITEHOUSE§

The Wistar Institute of Anatomy and Biology and Dept. of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia

That plant sterols may be absorbed by mammals was first suggested by Ellis and Gardner(1), who carried out experiments involving feeding of ordinary or ether-extracted feedstuffs to rabbits and found the liver sterol content of the group fed the untreated diet to be the greater. Since then, other workers, using more direct means, have demonstrated absorption of phytosterols such as sitosterol (2,3) and ergosterol(4,5) in a number of experimental animals. The metabolic pathway of plant sterols is of interest since they comprise a significant proportion of the sterol

* Presented in part at 5th Internat. Conf. on Biochemical Problems of Lipids, Marseilles, France, July 1960.

† We are greatly indebted to Drs. G. J. Alexander and W. G. Dauben for generous gifts of ergosterol-28-C¹⁴ and ergosterol-U-C¹⁴, respectively. We are also grateful to Leon Gogolski and John Langan for technical assistance. This work was supported, in part by a grant from Nat. Inst. Health, Bethesda, Md.

‡ This work was done during the tenure of an Established Investigatorship of American Heart Assn.

§ Present address: Dept. of Biochemistry, Univ. of Oxford, England.

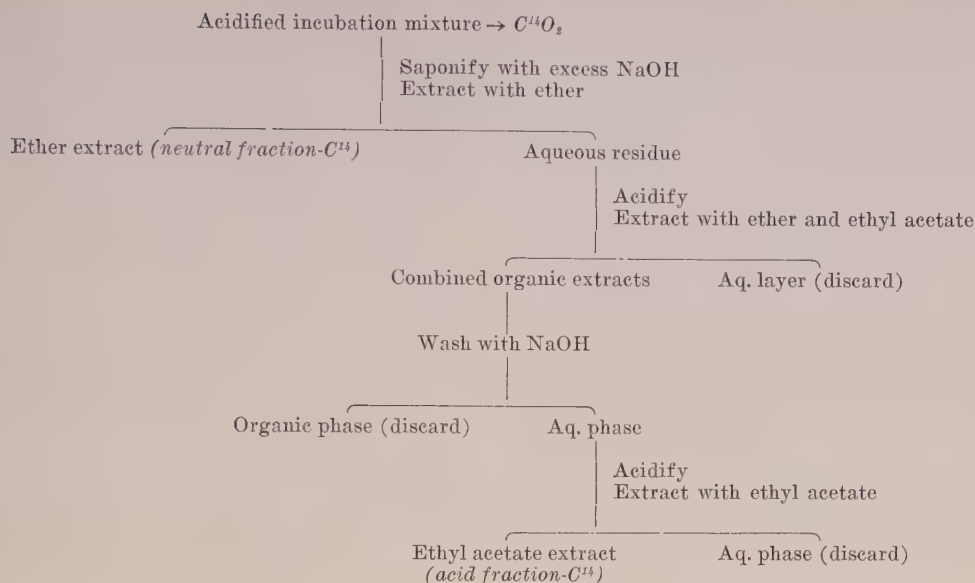


FIG. 1. Isolation of radioactive products from incubation of ergosterol- C^{14} with liver mitochondria.

content of the average human diet. The experiments described here have demonstrated that ergosterol is readily oxidized by enzymes present in rat and mouse liver mitochondria.

Methods. Ergosterol labelled with C^{14} was prepared biosynthetically from yeast grown in presence of formic acid- C^{14} , yielding ergosterol-28- C^{14} (6,7) or sodium acetate-1- C^{14} , yielding ergosterol-U- C^{14} (8) containing radioactivity at carbon atom 25 *inter locos* (8,9). Cholesterol-26- C^{14} was synthesized according to the procedure of Dauben and Bradlow (10). Cholesterol-4- C^{14} was purchased from the New England Nuclear Corp., Boston, Mass. The 3 α , 7 α , 12 α -trihydroxycoprostan-26-oic acid was isolated from alligator bile (11).

Mitochondria were isolated from the livers of male Wistar rats and Swiss Hygienic mice of both sexes. The procedure has been described in detail (12,13). The liver supernatant fraction (SF) was prepared by boiling the solution remaining after removal of all particles from a liver homogenate by centrifugation at $100,000 \times g$ for 25 minutes.

Incubations were carried out at $37^\circ C$ in stoppered 125 ml Erlenmeyer flasks with center wells containing 2.0 ml of 2.5N sodium hydroxide. Each flask contained washed

mitochondrial preparation (1 ml), a sterol emulsion in 0.25 M tris (hydroxymethyl) aminomethane HCl, pH 8.5 (5 ml) and one ml of a solution containing the following: diphosphopyridine nucleotide (5 mg), adenosine-5'-monophosphate (8 mg), adenosine triphosphate (25 mg), glutathione (15 mg), trisodium citrate monohydrate (30 mg), magnesium chloride (10 mg), potassium penicillin G (2000 U), and streptomycin sulfate (1 mg). In addition 5 ml of SF or of 10% (w/v) aqueous sucrose, was added to all incubations.

At the end of incubation period sulfuric acid was added and the flasks re-stoppered and shaken for 3 hours to displace carbon dioxide from the medium. The carbon dioxide trapped in the alkali present in the center well was precipitated as $BaC^{14}O_3$ with barium chloride. Radioactivity of the barium carbonate, suspended in a thixotropic gel (12,13), was assayed in a Packard Tri Carb liquid scintillation counter.

Neutral and acidic radioactive products were isolated from the incubation mixtures according to the scheme given in Fig. 1. The acidic material was chromatographed on paper according to the method of Beyreder and Rettenbacher-Daubner (14) using the

TABLE I. Oxidation of Ergosterol-28-C¹⁴ and Ergosterol-U-C¹⁴ by Rat and Mouse Liver Mitochondria.

Substrate	Mitochondria	% oxidation*				
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Ergosterol-28-C ¹⁴	Rat	3.6	2.4			
	Mouse	1.3	1.8			
Ergosterol-U-C ¹⁴	Rat	.7	2.2	.9	.6	2.7
	" †	(10.5)	(33.0)	(13.5)	(9.0)	(40.5)
	Mouse	.9	.9	.9		
	" †	(13.5)	(13.5)	(13.5)		

* Computed as BaC¹⁴O₃/substrate-C¹⁴.† Recalculated assuming C¹⁴O₂ arises only from C₂₅.

upper phase of the mixture glacial acetic acid:toluene:water—40:40:8 v/v as the solvent. For determination of R_f values each chromatogram was cut into 1 cm strips which were counted directly, in the presence of scintillation medium, in a liquid scintillation counter.

Results and discussion. Both ergosterol-28-C¹⁴ and ergosterol-U-C¹⁴ were oxidized by rat and mouse liver mitochondria yielding radioactive carbon dioxide (Table I). Parallel incubations without mitochondria or with boiled mitochondria gave no radioactive carbon dioxide. Inclusion of penicillin and streptomycin in all incubations strengthens the conclusion that ergosterol oxidation was due to the liver mitochondria and not to any contaminating bacteria.

The experimental conditions required for *in vitro* oxidation of ergosterol are similar to those under which the cholesterol side chain is oxidized(12,15). Especially noteworthy is the requirement for a thermostable factor (SF), present in the supernatant fraction of the liver homogenate, for optimal oxidation of both ergosterol and cholesterol (Table II). This factor is apparently not identical with TPNH, nor with any other known enzyme for steroid metabolism(15).

TABLE II. Influence of Supernatant Fraction (SF) on Sterol Oxidation by Rat and Mouse Liver Mitochondria.

Substrate	% oxidation*			
	Rat		Mouse	
	-SF	+SF	-SF	+SF
Ergosterol-28-C ¹⁴	.2	7.5	.7	3.3
Ergosterol-U-C ¹⁴	1.8	5.9	.4	4.9
Cholesterol-26-C ¹⁴	.1	7.9	.1	5.9

* Computed as BaC¹⁴O₃/substrate-C¹⁴.

Comparison of the relative yields of radioactive carbon dioxide at different time intervals from ergosterol-28-C¹⁴ and from ergosterol-U-C¹⁴ (labelled at C₂₅) suggests that C₂₈ may be cleaved before C₂₅ and the terminal isopropyl group (Table III). Since the C¹⁴-labelled steroids used in

TABLE III. Relative Rates of Sterol Oxidation by Rat Liver Mitochondria.

Substrate	% oxidation*		
	3 hr	6 hr	18 hr
Ergosterol-28-C ¹⁴	5.8	6.1	11.8
Ergosterol-U-C ¹⁴	.6	1.3	1.7
(Ergosterol-25-C ¹⁴)†	(9.0)	(19.5)	(25.5)
Cholesterol-26-C ¹⁴	1.7	5.3	17.1

* Computed as BaC¹⁴O₃/substrate-C¹⁴.† Recalculated assuming C¹⁴O₂ from ergosterol-U-C¹⁴ arises only from C₂₅.

these experiments were of different specific activities, no useful comparison can be made between absolute yields of radioactive carbon dioxide formed from the different substrates.

In an effort to obtain an indication of the type of acidic products formed, from both ergosterol-C¹⁴ substrates, all incubations were worked up according to the scheme given in Fig. 1. Whereas an appreciable amount of radioactivity was recovered from all incubations where ergosterol-U-C¹⁴ was the substrate, radioactivity was recovered in the acidic fraction of only one of the experiments with ergosterol-28-C¹⁴. The acidic fractions obtained were combined and chromatographed on paper. These results were compared with those obtained on chromatography of the acidic products isolated after incubations of cholesterol-4-C¹⁴ and cholesterol-26-C¹⁴ with rat and mouse liver mitochondria. Comparisons were also made with

TABLE IV. R_f Values of Radioactive Acidic Products Formed by Mitochondrial Oxidation of Sterols.*

Substrate	Mitochondria	R_f	
Cholesterol-26-C ¹⁴	Rat	.15	.94
	Mouse	.11	.93
Cholesterol-4-C ¹⁴	Rat	.80	.87
	Mouse	.11	.93
Ergosterol-U-C ¹⁴	Rat	.03	.88
	Mouse	.03	.88
Cholic acid	(Reference Cpd)	.82	
Trihydroxycoprostanic acid	(" ")		.89

* Paper chromatography according to Beyreder and Rettenbacher-Daubner(14).

chromatograms of cholic acid and trihydroxycoprostanic acid (THCA). The results are presented in Table IV.

In vivo experiments have shown that rat liver is able to oxidize several 27-carbon sterols to products resembling, but not identical with, the normal bile acids formed from cholesterol(16,17,18). Conversion of C₂₈ and C₂₉ sterols such as ergosterol and sitosterol to compounds resembling cholesterol has been adduced from experiments with beetles (19) and flies(20). That insects can metabolically alter the side chain and degree of unsaturation of sterols has been directly demonstrated in the conversion of ergosterol to 22-dehydrocholesterol by cockroaches(21). Hanahan(4) and Glover(5) have found that ergosterol is converted to acidic material, "pseudo" bile acid in nature, in the rat and guinea pig, respectively. Werbin *et al.*(22, 23) have presented evidence that, in the guinea pig, β -sitosterol is converted to cortisol, acidic products and, probably, cholesterol.

In animals, the principal pathway of cholesterol catabolism is through formation of bile acids by the liver(16). Our earlier experiments have shown that liver mitochondria readily oxidize the terminal carbon atoms of cholesterol. The experiments reported here indicate that ergosterol is degraded in a similar fashion with removal of the "extra" methyl group (C₂₈) and at least C₂₅ of the side chain, presumably as a consequence of the oxidation of the terminal isopropyl group. The many similarities between the oxidation of cholesterol and of ergosterol *in vitro* suggest that the enzyme systems

concerned are closely related to each other, if not identical.

Paper chromatography of the radioactive acidic products also indicates the similarity of the metabolites of cholesterol and ergosterol. Oxidation of cholesterol-4-C¹⁴ by rat liver mitochondria yields 2 radioactive compounds whose R_f values are similar to those of cholic and trihydroxycoprostanic acids. When incubated with mouse liver mitochondria, cholesterol-4-C¹⁴ yields 2 radioactive compounds, one slightly less polar and one much more polar than trihydroxycoprostanic acid. When the substrate is cholesterol-26-C¹⁴ both rat and mouse liver mitochondria yield radioactive compounds whose R_f values resemble the 2 obtained from oxidation of cholesterol-4-C¹⁴ by mouse liver mitochondria. When cholesterol-26-C¹⁴ is the substrate, no radioactive compound resembling cholic acid (in R_f value) is obtained, as would be expected, although such a compound could be detected by spray reagents (13). Oxidation of ergosterol-U-C¹⁴ by either rat or mouse liver mitochondria yields the same 2 radioactive acidic compounds. One is more polar than the most polar substance observed when cholesterol-C¹⁴ is the substrate; the other has an R_f value akin to that of trihydroxycoprostanic acid. Hoshita (24,25) has recently demonstrated that the acid isolated from toad bile by Shimizu and Oda(26) (trihydroxybufosteroholic acid) is 3 α , 7 α , 12 α -trihydroxy-24-methyl- Δ^{22} -coprostanic acid. If this bile acid may be regarded as a metabolite of ergosterol it is one in which ring saturation and hydroxylation similar to those observed in the initial stages

of cholesterol degradation have taken place. Such a compound could conceivably be that encountered by us in our chromatographic experiments.

The physiological significance of our observations is that plant sterols, absorbed from the intestine, can be degraded and presumably excreted *via* the bile in the form of water soluble acids.

Summary. Oxidation of ergosterol-28-C¹⁴ and of ergosterol-U-C¹⁴ by mitochondrial preparations from rat or mouse livers has been investigated. Either substrate yielded C¹⁴O₂. Ergosterol-U-C¹⁴ yielded radioactive neutral and acidic products, while ergosterol-28-C¹⁴ gave only neutral radioactive material. Among the acidic products obtained from ergosterol-U-C¹⁴ was a radioactive acid with R_f close to that of trihydroxycoprostanic acid. Similarities between the oxidation of cholesterol and ergosterol by liver mitochondria *in vitro* suggest that enzyme systems involved are closely related to each other, if not identical.

1. Ellis, G. W., Gardner, J. A., *Proc. Roy. Soc (London)* 1912, vB84, 461.
2. Gould, R. G., *Trans. N. Y. Acad. Sci.*, 1955, v18, 129.
3. Swell, L., Boiter, T. A., Field, H., Jr., Treadwell, C. R., *Nutrition*, 1956, v58, 385.
4. Hanahan, D. J., Wakil, S. J., *Arch. Biochem. Biophys.*, 1953, v44, 150.
5. Glover, J., Leat, W. M. F., Morton, R. A., *Biochem. J.*, 1957, v66, 214.
6. Danielsson, H., Bloch, K., *J. Am. Chem. Soc.*, 1957, v79, 500.
7. Alexander, G. J., Gold, A. M., Schwenk, E.,

J. Biol. Chem., 1958, v232, 599.

8. Hanahan, D. J., Wakil, S. J., *J. Am. Chem. Soc.*, 1953, v75, 273.

9. Dauben, W. G., Hutton, T. W., Boswell, G. A., *ibid.*, 1959, v81, 403.

10. Dauben, W. G., Bradlow, H. L., *ibid.*, 1958, v72, 4248.

11. Briggs, T., Whitehouse, M. W., Staple, E., *Arch. Biochem. Biophys.*, 1959, v85, 275.

12. Whitehouse, M. W., Staple, E., Gurin, S., *J. Biol. Chem.*, 1959, v234, 276.

13. Kritchevsky, D., Kolman, R. R., Whitehouse, M. W., Cottrell, M. C., Staple, E., *J. Lipid Research*, 1959, v1, 83.

14. Beyreder, J., Rettenbacher-Daubner, H., *Monatsh.*, 1953, v84, 99.

15. Whitehouse, M. W., Staple, E., Gurin, S., *J. Biol. Chem.*, 1961, v236, 73.

16. Bergström, S., *Record Chem. Progress* 1955, v16, 63.

17. Harold, F. M., Jayko, M. E., Chaikoff, I. L., *J. Biol. Chem.*, 1955, v216, 439.

18. Harold, F. M., Abraham, S., Chaikoff, I. L., *ibid.*, 1956, v221, 435.

19. Beck, S. D., Kapadia, G. G., *Science*, 1957, v126, 258.

20. Bergmann, E. D., Levinson, Z. H., *Nature*, 1958, v182, 723.

21. Clark, A. J., Bloch, K., *J. Biol. Chem.*, 1959, v234, 2589.

22. Werbin, H., Chaikoff, I. L., Jones, E. E., *ibid.*, 1959, v234, 282.

23. ———, *ibid.*, 1960, v235, 1629.

24. Hoshita, T., *J. Biochem. (Japan)*, 1959, v46, 507.

25. ———, *ibid.*, 1959, v46, 1551.

26. Shimizu, T., Oda, T., *Z. physiol. Chem.*, 1934, v227, 74.

Received January 6, 1961. P.S.E.B.M., 1961, v106.

Endocrine Studies of Chlordiazepoxide.* (26449)

ALFRED BORIS, JOSEPH COSTELLO, MARIE M. GOWER AND JOAN A. WELSCH
(Introduced by William Schallek)

Dept. of Pharmacology, Hoffmann-La Roche, Nutley, N. J.

Several reports in the literature indicate that psychotropic drugs of the phenothiazine and reserpine type have endocrine effects, particularly on the pituitary-gonadal axis

(1-6). We therefore decided to study the endocrine effects of chlordiazepoxide, a member of a new chemical class of compounds possessing potent pharmacological activity in animals(7-8) and psychotropic effects in man(9).

* Librium®, Hoffmann-La Roche.

TABLE I. Endocrine Studies of Chlordiazepoxide.

Test	No. animals per group	Initial body wt (g)	Dosage	Response
Reduction in testes and prostate wt in immature rats	7-10	50-60	2.5 or 5 mg/day s.e. or 50 mg/kg/day p.o. × 10 days	Negative
Prevention of ovarian hypertrophy in parabiotic rats	4-7 pairs	50-60	5 mg/day × 10 days s.e.	"
Interruption of estrous cycle in rat	7	250-260	50 or 100 mg/kg/day p.o. × 5 days	"
Prevention of ovulation in rabbit in response to mating	6	2800-3000	25 or 50 mg/kg p.o. 1 hr prior to mating	"
Prevention of goitrogenic activity of thiouracil	9-10	220-230	10 mg/day × 14 days s.e.	"
Effect upon seminal vesicle and prostate wt in castrated rats	8	50-60	2.5 mg/day × 7 days s.e. and/or testosterone propionate .05 mg/day × 7 days s.e.	"
Effect upon uterine wt in ovariectomized rats	10	85-90	5 or 10 mg/day × 3 days s.e. and/or estradiol benzoate .0005 mg/day × 3 days s.e.	"
Progestational and anti-progestational activity in estrogen primed, intact rabbit	4	800-900	5, 10, or 20 mg/day × 5 days p.o. and/or progesterone 0.2 mg/day × 5 days s.e.	"

Methods. Rats were obtained from Charles River Breeding Laboratories, Brookline, Mass. Subcutaneous (s.c.) injections were given in sesame oil, while distilled water served as the vehicle in oral (p.o.) administrations. Female rats were ovariectomized and united parabiotically with an intact female partner on the day following ovariectomy. The ovariectomized parabiont received all injections. Rats used for estrous cycle studies were preselected for normal cycling. Vaginal smears were examined once daily during treatment. Thiouracil was administered in the drinking water as an 0.1% solution. Treatment of male castrates started one day after castration. Ovariectomized rats were used 4 days following ovariectomy. Progestational activity was determined histologically in rabbits primed for 5 days before chlordiazepoxide treatment with .006 mg estradiol benzoate per day s.c.

Results and discussion. Table I indicates that chlordiazepoxide failed to manifest endocrine effects in any of the procedures employed at dosages which produce significant non-endocrine pharmacological effects in rats and other species (7-8). The lack of effect upon the pituitary-gonadal axis is particu-

larly evident, since chlordiazepoxide was inactive in 4 different tests utilizing 2 species. In parallel studies with potent gonadotropin inhibitors such as estradiol or testosterone, reduction in testes weight of 60 to 70% occurred with dosages of .005 mg and 0.1 mg/day, respectively, after 10 days administration to intact immature male rats.

The goitrogenic activity of thiouracil is dependent on increased output of thyrotropin from the pituitary, and thyroid enlargement does not occur in hypophysectomized, thiouracil-treated animals (10). Consequently, the failure of chlordiazepoxide to prevent thiouracil-induced thyroid enlargement indicates that chlordiazepoxide does not possess anti-thyrotropic activity.

Summary. Studies utilizing standard endocrine technics indicate that chlordiazepoxide does not possess gonadal hormone-like activity, anti-gonadal hormone activity, anti-goitrogenic activity, nor does it interfere with normal functioning of the pituitary-gonadal axis.

1. Sulman, F. G., *Arch. int. pharmacodyn.*, 1959, v118, 298.
2. ———, *ibid.*, 1959, v121, 85.
3. Khazan, N., Sulman, F. G., Winnik, H. Z.,

PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 201.

4. Erikson, L. B., Reynolds, S. R. M., DeFeo, V. J., *Endocrinology*, 1960, v66, 824.

5. Gaunt, R., Rinzi, A. A., Antonchak, H., Miller, G. J., Gilman, M., *Ann. N. Y. Acad. Sci.*, 1954, v59, 22.

6. Barraclough, C., *Fed. Proc.*, 1955, v14, 9.

7. Randall, L. O., Schallek, W., Heise, G. A., Keith, E. F., Bagdon, R. E., *J. Pharm. Exp. Ther.*, 1960,

v129, 163.

8. Randall, L. O., *Dis. Nerv. System*, 1960, v21 (Suppl. 7).

9. Tobin, J. M., Lewis, N. D. C., *J. Am. Med. Assn.*, 1960, v174, 96.

10. Mackenzie, C. G., Mackenzie, J. B., *Endocrinology*, 1943, v32, 185.

Received January 11, 1961. P.S.E.B.M., 1961, v106.

Serological Relationships between Collagenase and the A₂-Isoagglutinin-like Substance of Animal Parasites.* (26450)

JOSÉ OLIVER-GONZÁLEZ AND H. NAIM KENT

Departments of Microbiology, School of Medicine, San Juan, P. R., and Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md.

Recent studies have indicated that the A₂-isoagglutinin-like substance (A₂IS) in animal parasites may be associated with a collagenase complex. Thus the sera from rabbits artificially immunized against the A₂IS agglutinate human erythrocytes of Group O treated with tannic acid and exposed to collagenase prepared from various sources(1). Also, collagenase, like A₂IS inhibits the a₂ isoagglutinins in human sera of Groups B and O. The A₂IS utilized in these studies was extracted from the cuticle of *Ascaris* and was used as a crude material. A purified A₂IS has been prepared which shows more activity than the crude material with respect to inhibition of a₂ isoagglutinins and which is also serologically related to collagenase.

Lewert *et al.*(2) have shown that collagenase-like enzymes are present in the larval forms of *Schistosoma mansoni*, *Ancylostoma caninum* and *Strongyloides ratti*. Collagen-like substances have also been described from various animal parasites(3,4) particularly from the cuticle of *Ascaris*, which constitutes a good source of A₂IS. Collagenase, therefore, is apparently present in animal parasites either as a component of their tissues or in their metabolic products.

Materials and methods. Animal inoculations. Male rabbits weighing from 1.5 to 2

kilos were artificially immunized with various substances as described previously(1). Dogs weighing 4.5 to 5 kilos were each injected intravenously with 2 to 3 mg of A₂IS, collagenase, pepsin and trypsin[†] per kilo of body weight. The animals were observed closely for reactions to the various substances and were bled before injection and immediately after death. Dogs were autopsied and tissues fixed for histopathological studies immediately after death.

Preparation of purified A₂IS. The crude and dry material obtained from *Ascaris* cuticle, according to the procedure described elsewhere(1) was reextracted with triple distilled water at 4°C, centrifuged for 30 minutes at 9000 × G (International refrigerated centrifuge PR-1) and the supernatant dialyzed exhaustively against triple distilled water in a cold room for a period of 2 days with several changes. The dialyzed solution was then lyophilized. The lyophilized material will be referred to as "purified A₂IS".

Total protein analyses were carried out by the method of Lowry *et al.*(5); total carbohydrate and cold trichloroacetic acid-precipi-

[†] Collagenase A—Nutritional Biochemical Corp., Cleveland, Ohio.

Collagenase B—Mann Research Laboratories, New York.

Pepsin and Trypsin—Difco Laboratories, Inc., Detroit, Mich.

* These investigations were supported by grants from Nat. Inst. Health, U.S.P.H.S., Bethesda, Md.

table-protein-bound-carbohydrates were determined colorimetrically according to Siebert(6). Electrophoresis was performed with "OXOID" Cellulose acetate strips, as described by Kohn(7).

Agglutination tests. The substances to be tested for inhibition of the a_2 isoagglutinins in human sera of Groups B and O were first dissolved in the specific pH buffered saline for each substance, then diluted 2-fold in 0.1 cc volumes of 0.85% sodium chloride. One-tenth cc volume of Group O or B human sera was then added to the 0.1 cc volume of the respective dilution of substance, and incubated at 37°C for 30 minutes. Each serum-substance mixture was then diluted 2-fold, after which 0.1 cc of a 2% suspension of washed human erythrocytes of sub-group A_2 was added. The serum erythrocyte mixture was left at room temperature for 1 hour and examined for agglutinated erythrocytes under the stereoscope at $\times 20$. Substances tested for a_2 inhibitory activity were crude and purified A_2 IS, collagenase, pepsin and trypsin.

Sera from dogs injected with various substances were tested for agglutinins against their own erythrocytes by mixing 0.1 cc volumes with 0.5 cc of a 2% suspension of the dogs' own erythrocytes before and after injection which had been washed 3 times in saline. The mixtures in tubes were incubated at 37°C for 60 minutes, then portions were examined under the microscope at $\times 340$.

Hemagglutination tests were performed on human and animal sera following Boyden's technic(8), with slight modifications, as used by Kagan(9) in his studies concerning serological diagnosis of helminthic infections. Ten mg of the enzymes collagenase, trypsin and pepsin were dissolved in 10 cc of buffered saline at pH 8.8 and 5.5 respectively. The Group O human erythrocytes treated with tannic acid were exposed for 10 to 15 minutes to the above concentration of substances or to further dilutions, if necessary. Reference positive sera were obtained from rabbits immunized against A_2 IS. As a daily procedure, known positive and negative sera were tested against Group O

tannic acid treated erythrocytes and exposed to the antigens. If the tests were positive to high titers with the known positive, and no agglutination was observed with the known negative sera, all of the remaining unknown sera were then tested. Sera from rabbits inoculated with A_2 IS were tested against O-collagenase, O-trypsin and O-pepsin, and with the homologous O- A_2 IS erythrocytes as well.

The sera of rabbits immunized against A_2 IS were treated with powdered collagenase, trypsin, pepsin and crude A_2 IS, and were then tested for agglutinins against O- A_2 IS erythrocytes. Five milligrams of the substance was added to 0.5 cc of the serum mixed well by means of a 1 cc tissue homogenizer, then incubated at 37°C for one hour. After incubation the mixture was centrifuged for 5 minutes at $1200 \times g$. Treated and untreated sera were then tested for agglutinins against O- A_2 IS erythrocytes.

Agar gel double diffusion tests were performed using the rapid slide technic described by Yakulis and Heller(10). Sera from rabbits immunized against collagenase were tested against a 1:1,000 solution of the purified A_2 IS and of collagenase. Normal rabbit sera were also tested against collagenase as controls. The slides were incubated at 37°C for several days and examined macroscopically for presence of bands of precipitate.

Results. Biochemical characteristics of the purified A_2 IS. Preliminary determinations have revealed that only 22% of the crude material from the cuticle was water-soluble under the experimental conditions described. The electrophoretic patterns of purified A_2 IS indicated the presence of one slow-moving homogeneous protein (Fig. 1a) accompanied by a negligible impurity. Under the same electrophoretic conditions, the pattern of commercial collagenase showed mainly 2 fractions (Fig. 1b): the slow-moving one corresponding to the A_2 IS protein. Therefore the electrophoretic similarities at pH 8.6 of these 2 proteins are apparent.

The gross chemical composition of the A_2 IS protein is shown in Table I. The high content of carbohydrate in A_2 IS suggests that it is a glycoprotein.

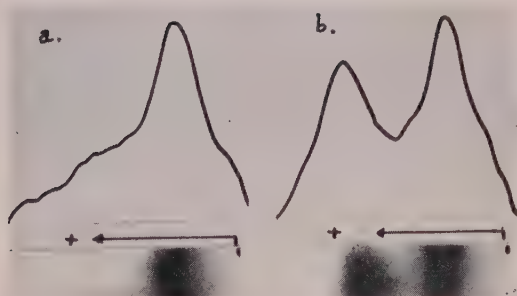


FIG. 1. a and b. Electrophoretic patterns of purified A₂IS and of collagenase showing similar slow-moving protein fractions.

Inhibition of α_2 isoagglutinins by various substances. Of all substances tested, collagenase and the purified A₂IS were the ones which inhibited the α_2 isoagglutinins in human sera of Groups B and O, when using the lowest concentration of material. Activity of the collagenase preparations varied from 4 to 20 μ g per cc of serum. That of purified A₂IS was similar to collagenase, 4 μ g per ml of serum. Pepsin and crude A₂IS inhibited at a level of 350 and 2500 μ g, respectively, while trypsin had no effect (Table II).

TABLE I. Chemical Composition of Purified A₂IS.

Total protein	Total carbohydrate	Protein-bound carbohydrate	Free carbohydrate
% W/W*			
17	83	66.3	22.7

* Expressed as % of dry wt.

Hemagglutination reactions with rabbit antisera. Sera from rabbits immunized against A₂IS reacted to high titers with the homologous O-A₂IS erythrocytes and with O-collagenase, but not with O-pepsin or O-trypsin

TABLE II. Inhibition of α_2 Isoagglutinins in Human Serums of Groups B and O by Various Substances.

Enzyme or substance used	α_2 isoagglutinin titer		
	Before treatment	After treatment μ g/cc of serum	
Crude A ₂ IS	1:128	2,500	0
Purified A ₂ IS	1:128	4	0
Collagenase A	1:128	4	0
B	1:128	20	0
Pepsin	1:128	350	0
Trypsin	1:128	3,000	1:128
"	1:128	5,000	1:128

treated erythrocytes. Treatment of these sera with A₂IS and with collagenase absorbed the antibody completely. Treatment of the sera with trypsin resulted in partial absorption, but there was no absorption with pepsin (Table III).

TABLE III. Absorption of Hemagglutinins by Various Substances.

Substance	Hemagglutinin titer against O-A ₂ IS erythrocytes	
	Before treatment of serum	After treatment of serum
Collagenase A	1:20,480	0
Crude A ₂ IS	1:40,960	0
Purified A ₂ IS	1:40,960	0
Pepsin	1:10,240	1:10,240
Trypsin	1:40,960	1:1,280

Agar gel precipitin reactions. The rabbit anticollagenase sera reacted with a 1:1,000 suspension of collagenase in saline as shown by the presence of one thin and one coarse band of precipitate. These antisera also reacted with the 1:1,000 solution of purified A₂IS from *Ascaris* cuticle. One major band could be clearly seen which coalesces with the coarse band formed with collagenase, (Fig. 2). Normal rabbit sera did not react with collagenase or with the purified A₂IS.

Reactions to intravenous injection. The dogs which received injections of crude A₂IS and of collagenase died within 4 hours thereafter. The animals manifested symptoms of an anaphylactoid reaction, such as urination, defecation, vomiting and death. Urine and feces of the animals injected with collagenase were of a dark red color, indicating presence of blood. Sera from these dogs had agglutinins against the animals' own erythrocytes. Agglutinins in animals injected with collagenase were higher than those injected with A₂IS (1:32 dilution of serum as compared with 1:2). Dogs injected with trypsin and pepsin did not manifest anaphylactoid reactions, and survived.

Histopathological studies of liver and spleen of dogs injected with A₂IS and collagenase also revealed marked congestion and agglutinated erythrocytes. Observations on changes in the kidneys and other organs will be reported later.

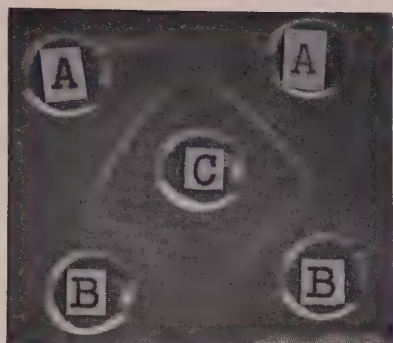


FIG. 2. Agar gel diffusion slide showing precipitin bands formed when anticollagenase sera (C) reacted against A₂IS (A) and collagenase (B).

Discussion. These results indicate that the A₂IS from animal parasites is serologically related to collagenase. Rabbit antisera against A₂IS reacted positively with O cells coated with collagenase. No such reactions were observed with these sera against O cells coated with pepsin or trypsin. Furthermore, treatment of the rabbit antisera with collagenase absorbed the hemagglutinins for O-A₂IS erythrocytes.

Further evidence for the serological relationship between the A₂IS substance and collagenase is seen in the agar gel double diffusion tests. The purified A₂IS formed 2 bands of precipitate when tested against the anticollagenase sera. The most prominent band coalesces with the coarse band observed when the same sera are tested against the homologous antigen collagenase.

Another relationship is indicated by the electrophoretic patterns of both substances, which shows a similar slow moving component.

Close serological relationships between A₂IS and collagenase are thus indicated by: 1. Specific action and degree of inhibitory activity against α_2 isoagglutinins in human sera of Groups O and B. 2. Cross reactions as observed in hemagglutination tests using A₂IS rabbit antisera against Group O-collagenase erythrocytes. Also the antibody against each substance was absorbed by the heterologous as well as by the homologous antigen. 3. Cross precipitin reactions observed in agar diffusion tests when anticollagenase rabbit sera are tested against the A₂IS. 4. Death of dogs when both sub-

stances are injected intravenously. The animals die from an anaphylactoid reaction with similar reactions and histopathological observations.

Collagenase, as prepared commercially from species of *Clostridia* is not a pure substance and may have other active impurities. This is shown by the fact that 2 bands of precipitate are observed when tested against homologous antisera. One of the bands is coarse, which is the one coalescing with the major band formed when the serum is tested against the purified A₂IS. This indicates that the A₂IS may be present as part of the collagenase complex or as a contamination. The serological relationships discussed above indicate, however, that A₂IS may be an important part of the collagenase complex. Further studies on the antigenic and enzymatic properties of collagenase and A₂IS will undoubtedly clarify this aspect.

The presence of related antigenic substances such as the A₂IS found in *Ascaris* and in *Clostridia* is an illustration of a common antigen between helminths and bacteria which may be important in serological diagnosis and host's reactions.

Death occurs when dogs are injected intravenously with collagenase or A₂IS. The mechanism of death is not understood, although autoagglutination of erythrocytes may be a factor. Apparently the substance adsorbs *in vivo* onto the dogs' red cells and thus a reaction occurs between the α_2 agglutinins in the dog plasma and the dogs' erythrocytes. The erythrocytes apparently change in antigenicity as a result of adsorption of the new antigen. This is indicated by the fact that the serum agglutinates erythrocytes obtained after inoculation of the A₂IS and collagenase and not those obtained before inoculation. *In vivo* adsorption of collagenase or like substances onto erythrocytes may take place during infection with organisms which secrete this substance. This may be the mechanism for autoagglutination of erythrocytes observed during some infectious processes.

The anaphylactoid reaction observed in dogs after intravenous inoculation of collagenase and of A₂IS suggests that these sub-

stances are concerned in hypersensitivity reactions, in which autoagglutination of erythrocytes and other pathological phenomena caused by allergens are involved.

Summary. The A₂ isoagglutinin-like substance (A₂IS) from animal parasites has been prepared in a purified form from crude extracts of the cuticle of *Ascaris lumbricoides* var suum. Preliminary chemical studies indicated that this substance is a glycoprotein. The A₂IS is related to collagenase or a collagenase complex, as shown by cross hemagglutination reactions between anticollagenase sera prepared in rabbits, and the A₂IS substances adsorbed onto tannic acid treated Group O human erythrocytes. Also A₂IS reacts with anticollagenase sera in agar diffusion slides with formation of one band of precipitate. The two substances are also immunologically related as shown by the fact that they inhibit the α_2 isoagglutinins in human serum of Groups O and B when comparable amount of each substance is used

per ml of serum. A₂IS and collagenase also cause anaphylactoid symptoms and death when injected intravenously into dogs. It is suggested that collagenase or substances in the collagenase complex are present in infectious organisms which may be associated with hypersensitivity.

1. Oliver-González, J., *J. Infect. Dis.*, 1960, v107, 94.
2. Lewert, R. M., Lee, Chang-Ling, *Am. J. Trop. Med. Hyg.*, 1957, v6, 473.
3. Chitwood, B. G., *Proc. Helminthol. Soc.*, 1938, v3, 39.
4. Bird, A. F., *Exp. Parasitol.*, 1957, v6, 383.
5. Lowry, O. H., Rosebrough, N. J., Farr, L. A., *J. Biol. Chem.*, 1951, v193, 325.
6. Siebert, F. B., *ibid.*, 1946, v163, 511.
7. Kohn, J., *Biochem. J.*, 1957, v65, 9.
8. Boyden, S. V., *J. Exp. Med.*, 1951, v93, 107.
9. Kagan, I., *J. Immunol.*, 1958, v80, 396.
10. Yakulis, J. V., Heller, P., *Am. J. Clin. Path.*, 1959, 31, No. 4.

Received January 18, 1961. P.S.E.B.M., 1961, v106.

Renal Na-Reabsorption and O₂-Uptake in Dogs During Hypoxia and Hydrochlorothiazide Infusion. (26451)

KLAUS THURAU* (Introduced by Donald E. Gregg)

(With the technical assistance of Calvin Ervin and William Anderson, Jr.)

Department of Cardiorespiratory Diseases, Walter Reed Army Institute of Research, Washington, D.C.

It seems well established that sodium reabsorption in the kidney is an active process, which requires the bulk of the oxygen consumed by the kidney. In recent reports of experiments on the dog kidney, it has been shown that there is a fixed relation between sodium reabsorption and oxygen consumption (mEq Na/net mmol O₂). Thaysen, *et al.*, (1) using different dogs with large individual variations in rate of sodium reabsorption, found a fairly constant Na/net O₂ ratio of 28.5. Following acute hemorrhage, so that no glomerular filtration and, therefore, no sodium reabsorption occurred, basal

renal oxygen consumption was determined to be 1.0 μ mol/g kidney/min. Deetjen and Kramer (2) found a ratio of 24 over a wide range of sodium reabsorption in the same dog. By compressing the aorta above the renal artery, they were able to decrease the tubular sodium load by decreasing the glomerular filtration rate with a resulting drop in sodium reabsorption. By extrapolating from these data, basal renal oxygen consumption, *i.e.*, when no sodium is reabsorbed, was calculated to be 1.5 μ mol/g kidney/min.

The present study was undertaken with the following aims: (1) to decrease active reabsorption of sodium and oxygen consumption in the kidney by means of low arterial oxygen pressure or administration of hydro-

*U. S. Public Health Service Postdoctoral Research Fellow. Present address: Dept. of Physiology, Goettingen Univ., Goettingen, Germany.

chlorothiazide; (2) to determine the Na/net O₂ ratio under these conditions when tubular load and renal blood flow were essentially unchanged; and (3) to gain information about basal renal oxygen consumption under the influence of hypoxia or hydrochlorothiazide.

Twelve normal hydrated mongrels were anesthetized with nembutal (9 dogs) or with inactin[†] (3 dogs). A tracheal tube was inserted. After a left flank incision, a sinusoidal electromagnetic flow meter was placed around the unopened left renal artery for continuous measurement of total renal blood flow. The flow meter amplifier was a modified Kolin type(3). Mechanical zero for the flow meter was established either by cross-clamping the renal artery between flow meter and kidney for 2 to 5 sec., or by intravenous injection of 75 µg of epinephrine at the end of experiment. The deflections recorded by either method were identical. Femoral arterial pressure (using strain gauge) and renal blood flow were recorded on an Electronics for Medicine-DR8. Blood samples were collected by catheters inserted in the brachial artery and renal vein (*via* spermatic vein). The left ureter was cannulated close to the renal pelvis for urine samples. Renal oxygen uptake was calculated from renal blood flow (RBF) and renal A-V oxygen difference (Van Slyke-Neill). The creatinine infusion technic was applied for determination of glomerular filtration rate (GFR) (Beckman spectrophotometer). Amount of reabsorbed sodium was calculated as the difference between tubular load and excretion (flame photometer, Perkins Elmer). Serum and urine pH were measured with a Cambridge pH meter, and osmolalities in serum and urine by a Fiske osmometer. Hypoxia was produced by connecting the tracheal tube to a reservoir containing a gas mixture of 7.2% oxygen and 5% CO₂ in N₂. The arterial oxygen saturation under these conditions fell to an average of 35%. After a priming dose of 50 mg *i.v.*, hydrochlorothiazide was infused at a rate of 50 mg/h while the dogs were breathing room-air. In the hypoxic, as

TABLE I. Effect of Hypoxia and Hydrochlorothiazide upon Renal Hemodynamics, O₂ Consumption and Excretion of Water and Solutes.

	Control	Hypoxia	Hydrochlorothiazide
BP (mean), mm Hg	125	133	120
RBF, ml/min. g	4.84	4.95	3.9
GFR, ml/min. g	.81	.76	.71
Na load, µeq/min. g	126	117	105
Na excretion, % of load	1.19	1.55	4.91
O ₂ -consumption, µmol/min. g	5.6	4.95	4.37
UV, µl/min. g	16	22.4	31.7
Urine pH	6.65	6.67	—
Plasma pH	7.45	7.50	—
U/P osm	2.96	2.1	2.01
U/P creat.	76.02	53.02	24.41
C _{osm} , µl/min. g	47.5	47.0	63.7
T _{H₂O} ^c , µl/min. g	31.5	24.6	32.0
Na/net O ₂ ratio	28.6	31.1	32.0

well as in the hydrochlorothiazide group, the samples were taken after an initial 20 to 40 minute period of equilibration.

The average of the results is shown in Table I. In hypoxia, the arterial blood pressure and RBF are nearly unchanged but there is a slight decrease in GFR and tubular sodium load. In spite of the nearly unchanged tubular load, excreted sodium, expressed in per cent of tubular load, is increased. There is a concomitant decrease in oxygen consumption during hypoxia. In addition an increase in urine volume occurs with decreases in U/P osm and U/P creat. Serum pH and urine pH are nearly unchanged. Osmotic clearance is slightly elevated. T_{H₂O}^c falls from 31.5 to 24.6 µl/g kidney · min. The changes of renal function under hypoxia are reversible since after 20 minutes of room-air breathing control values were obtained.

Under the influence of hydrochlorothiazide, the RBF is reduced from 4.8 to 3.9 ml blood/g kidney · min, with a simultaneous decrease in GFR as well as in tubular sodium load. The per cent of filtered sodium excreted goes up from 1.19 to 4.91%. Renal oxygen consumption during hydrochlorothiazide infusion is the lowest in the individual experi-

[†] Na-ethyl-(1-methyl-propyl)-malonyl-thio-urea, Promonta GmbH, Hamburg, Germany.

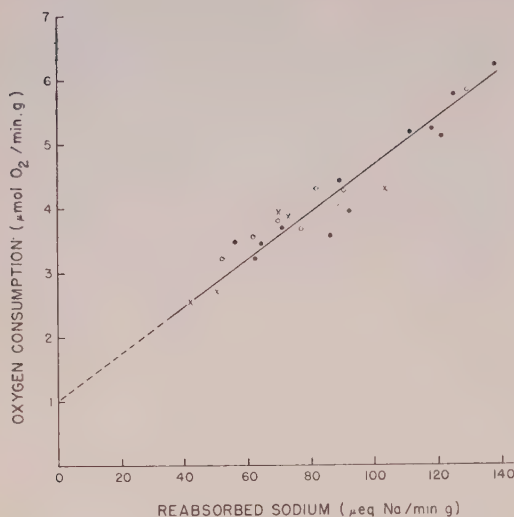


FIG. 1. Renal oxygen consumption and sodium reabsorption in dogs. ●, control; ○, hypoxia; ×, hydrochlorothiazide.

ment, as well as in the group average, when compared to control and hypoxia. Urine volume increases, as expected, with a concomitant decrease in U/P osm. and U/P creat. C_{osm} goes up from 47.5 to 65.2 $\mu\text{l/g kidney} \cdot \text{min}$. $T_{H_2O}^c$ remains essentially unchanged.

In Fig. 1 Na-reabsorption of the individual experiments is plotted against oxygen consumption. A line drawn through the middle of the individual points intercepts the oxygen consumption axis at 1 $\mu\text{mol oxygen/g kidney} \cdot \text{min}$, indicating basal renal oxygen consumption. There is no systematic deviation of any of the groups (control, hypoxia, hydrochlorothiazide) from this line.

In the last column of Table I the ratios Na/net O₂ are calculated for the 3 different groups (net O₂ = total oxygen consumption — basal renal oxygen consumption).

The present data suggest that active sodium reabsorption and net oxygen consumption in the dog kidney can be lowered proportionately during severe hypoxia. The fact that the alterations caused by hypoxia are abolished within 20 minutes after the dogs were again breathing room-air makes it unlikely that under these circumstances hormonal influences were involved. Furthermore, a respiratory alkalosis, which *per se* decreases sodium reabsorption, did not occur.

The decrease in $T_{H_2O}^c$ indicates that the efficiency of the terminal concentrating mechanism in the medulla is diminished by hypoxia. Although it cannot be determined with certainty, this diminution in $T_{H_2O}^c$ may be caused by a decrease of active sodium reabsorption in the medulla.

Na/net O₂ ratios in all 3 groups are in the same range, *i.e.*, between 28.6 and 32. The differences are not significant. They are slightly higher than the results of Deetjen and Kramer, but in accordance with Thyssen *et al.* Thus, it seems to be well established that a fixed ratio exists in the mammalian kidney which is higher than the fixed ratio of 16-20 in the frog skin or toad bladder(4,5,6). In contrast to the frog skin or toad bladder studies where only active sodium transport was measured, the method used deriving this ratio in the mammalian kidney has not excluded the influence of non-active sodium transport. Thus it may be that the ratio for active transported sodium in the mammalian kidney is somewhat lower.

The fact that hypoxic as well as hydrochlorothiazide values do not deviate systematically from the midline (Fig. 1) indicates that basal oxygen consumption of 1 $\mu\text{mol/g kidney} \cdot \text{min}$ is affected neither by hypoxia nor by hydrochlorothiazide.

Since renal sodium reabsorption depends upon tubular load and tubular reabsorptive activity, it is evident that primary changes in renal blood flow or filtration fraction *i.e.*, changes in GFR—as well as hormonal or drug influences upon the sodium reabsorptive mechanisms, determine renal oxygen consumption. The absence of a decrease in $T_{H_2O}^c$ to hydrochlorothiazide makes it unlikely that this drug affects sodium reabsorption in the medulla.

Summary. In anesthetized dogs renal oxygen consumption and sodium reabsorption were determined during hypoxia and under the influence of hydrochlorothiazide. In hypoxia (arterial O₂ saturation 35%) less sodium is reabsorbed and renal O₂ consumption is diminished. Urine volume is increased with a decrease in U/P_{osm}, U/P_{creat} and $T_{H_2O}^c$. During administration of hydro-

chlorothiazide both sodium reabsorption and O_2 consumption are lowered. Urine volume and C_{osm} are elevated, U/P_{osm} and U/P_{Creat} decreased while $T_{H_2O}^c$ remains unchanged.

Plotting Na reabsorption ($\mu eq/g \text{ min}$) against O_2 consumption ($\mu mol/g \text{ min}$) results in a straight line which intercepts the O_2 consumption axis at $1 \mu mol/g \text{ min}$. We believe that this value may be equated with renal basal O_2 consumption; *i.e.*, without Na reabsorption. The O_2 requirement for renal sodium reabsorption is calculated to be $28.6-$

$32 \mu eq \text{ Na}/\mu mol \text{ O}_2$.

1. Thaysen, J. H., Lassen, N. A., Munck, O., *Scand. Physiol. Congress*, Helsinki, 1959.
2. Deetjen, P., Kramer, K., *Klin. Wschr.*, 1960, v38, 680.
3. Khouri, E., Gregg, D. E., Hall, R., Rayford, C. R., *Physiologist*, 1960, v3, 93.
4. Zerahn, K., *Acta Physiol. Scand.*, 1957, v36, 300.
5. Leaf, A., Page, L. B., Anderson, J., *J. Biol. Chem.*, 1959, v234, 1625.
6. Leaf, A., Dempsey, E., *ibid.*, 1960, v235, 2160.

Received November 14, 1960. P.S.E.B.M., 1961, v106.

Studies of Acute Respiratory Illnesses Caused by Respiratory Syncytial Virus. I. Laboratory Findings in 109 Cases. (26452)

V. V. HAMPARIAN, A. KETLER, M. R. HILLEMANN, C. M. REILLY, L. MCCLELLAND, D. CORNFELD AND J. STOKES, JR.

Division of Virus and Tissue Culture Research, Merck Institute for Therapeutic Research, West Point, Pa., The Children's Hospital of Philadelphia, and the Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia

Morris *et al.*(1), described the first recovery of a virus, termed chimpanzee coryza agent (CCA), from the respiratory tract of chimpanzees with an acute respiratory illness and from a laboratory worker who had been in contact with these animals. The infected chimpanzees and the laboratory worker developed complement fixing and neutralizing antibodies in response to their infections. Chanock *et al.*(2,3) reported recovery of a similar virus from 2 infants with lower respiratory tract infection and showed antibody titer increases among persons with respiratory illness as well as controls; these workers were not able, however, to provide definitive evidence for etiologic association between the virus and human illness. Chanock renamed this agent the respiratory syncytial virus. Since that time, Rowe *et al.*(4) have reported positive laboratory diagnostic findings for the respiratory syncytial agent in an infant with pneumonia. More recently, Beem *et al.*(5) have recorded laboratory findings in 41 cases of respiratory illness among children infected with the respiratory syncytial agent.

Since 1959, our clinical-laboratory groups have engaged in extensive investigations of

the etiology and epidemiology of acute respiratory illness among patients admitted to the wards or to the outpatient clinics of the Children's Hospital of Philadelphia or the Hospital of the University of Pennsylvania. The present report describes the laboratory findings among 109 cases of acute respiratory illness shown to be caused by the respiratory syncytial virus. Subsequent reports(6,7) in this series describe the epidemiology of infections with the virus and the spectrum of clinical findings among patients with illnesses caused by this agent.

Materials and methods. Clinical population. The patients were children less than 8 years of age seen in outpatient clinics or admitted to wards of the Children's Hospital of Philadelphia or the Hospital of the University of Pennsylvania, and selected on the basis of having an acute respiratory illness of 4 days or less duration judged on clinical grounds to be of probable viral etiology. The majority of persons were from a low socioeconomic group, mostly Negro, and scattered geographically over a large area of Philadelphia. The cases described occurred during the period from Oct. 1959 through June

1960. Altogether, 563 cases of respiratory illness were studied. *Collection of specimens.* When seen initially, each child was examined clinically and 2 dry cotton swab samples were collected from the throat. These were extracted immediately into 5 ml of Difco veal infusion broth in a screw-cap tube, frozen in dry ice and transmitted to the laboratory where they were thawed, distributed and stored frozen in dry ice in flame-sealed glass containers until tested up to 1 year later. A 12 ml sample of blood was taken at the first visit and, routinely, 21 days later although there was delay in some instances owing to failure of the patients to return at the designated time. The sera were stored frozen at -20°C until tested up to 1 year later. Routine bacterial cultures of the throat failed to reveal primary bacterial etiology in any of the cases selected for study.

Laboratory. Tissue cultures. HeLa cell cultures were grown in Eagle's basal medium (8) with 10% inactivated normal horse serum and the WISH continuous cell line of human amnion (9) was propagated in Eagle's medium containing 10% inactivated normal calf serum. During virus propagation, all cell cultures were maintained using Eagle's basal medium containing 5% inactivated horse serum. Penicillin in a concentration of 100 units per ml and streptomycin in a concentration of 100 μg per ml were included in all tissue culture media. When cultures were inoculated with respiratory secretions, 50 units of mycostatin and 100 μg of neomycin sulfate /ml were incorporated into the maintenance medium. All cultures were incubated at 36°C . *Virus strains.* The Long strain of respiratory syncytial virus, originally recovered by Chanock *et al.* (2,3), was brought by one of us (M.R.H.) to this laboratory from the Walter Reed Army Inst. of Research. *Antisera.* Acute and convalescent sera from a human case of respiratory syncytial virus infection, originally obtained from Dr. J. A. Morris, was brought to this laboratory from the Walter Reed Army Inst. of Research. Rabbit antiserum against the Long strain was obtained from Dr. R. M. Chanock. Paired sera from proved cases of respiratory syncytial virus infection were used for virus iden-

tification purpose. *Virus recovery.* HeLa or human amnion (WISH) cell cultures were inoculated, each in triplicate, with 0.25 ml amounts of the thawed samples of respiratory secretions from each patient. The cultures were observed daily for cytopathic effect for 9 days, being refed with maintenance medium every 3 or 4 days. A single blind passage was made of all cultures which were negative. Tubes showing a definite cytopathic effect were harvested and the contents frozen. One or 2 subsequent passages were necessary to provide enough material for identification. *Viral infectivity assays.* All virus infectivity titrations were performed using serial 10-fold dilutions of virus. The inoculum dose was 0.2 ml per tube containing 2.0 ml of maintenance medium. The titer was the highest initial dilution of virus causing 50% or more degeneration of the cell sheet by the seventh day post inoculation. *Complement fixation (CF) tests.* The diagnostic CF antigen consisted of fluids from HeLa cell cultures infected with the Long strain of respiratory syncytial virus which were frozen, thawed and harvested when the cell sheets were completely degenerated. Uninfected normal HeLa culture fluids, similarly prepared, were used for control purpose. Serial 2-fold dilutions of inactivated sera in 0.1 ml volume were tested using 4 units of antigen in 0.1 ml volume and 2 exact units of complement in 0.2 ml. Fixation was overnight at 4°C and the hemolytic system consisted of 0.2 ml of a 1% sensitized sheep erythrocyte suspension. The titer was the highest initial dilution of serum giving less than 25% hemolysis. The complement was standardized in the presence of antigen and was free of antibody against the respiratory syncytial virus. *Serum neutralization (SN) tests.* In the tests, serial 2-fold dilutions of the inactivated sera were incubated for $\frac{1}{2}$ hour at room temperature with an equal volume of virus diluted to contain approximately 200 TCD₅₀ per 0.2 ml. HeLa cell cultures, in duplicate, were inoculated with 0.2 ml of the virus-serum mixtures and the tests were read on the third or fourth day of incubation when the control tubes showed specific viral degeneration of 50 to 75% of the cell sheet. The titer was the

TABLE I. Diagnostic Test Results Obtained in Representative Cases of Respiratory Syncytial Virus Infection.

Age	Patient	Laboratory findings				
		CF		Neutralization		Virus recovery
	Clinical diagnosis	Acute	Conv.	Acute	Conv.	
3 yr	Upper resp. illness	0*	320	0*	40	Positive
1 "	" " "	0	80	0	80	"
3 "	" " "	0	80	0	20	"
3 "	Bronchitis	0	320	0	160	"
3 "	"	0	160	0	20	"
2 "	"	10	320	0	80	"
6 mo	Bronchiolitis	0	40	0	10	"
8 "	"	0	40	0	20	"
1 yr	"	0	320	0	80	"
6 "	Bronchopneumonia	10	320	10	160	"
3 "	"	0	320	0	40	Negative
6 "	"	20	320	<20	640	"

* 0 titer = <1:5.

highest initial dilution of serum which caused complete suppression of viral degeneration. *Identification of viral isolates.* Isolates were identified both by the CF and serum neutralization technics using paired sera from human cases of respiratory syncytial virus infection, including the paired sera from the first reported human case of infection(1) with this virus. All identifications were controlled in simultaneous tests using the Long strain of respiratory syncytial virus. Tests with known positive Myxovirus parainfluenza 1, 2 and 3, measles and adenovirus group antisera gave negative results providing an additional control. *Results. Representative diagnostic test results.* Table I and Fig. 1 present representative serologic (CF and neutralization) and virus recovery findings among 34 cases of respiratory illness caused by the respira-

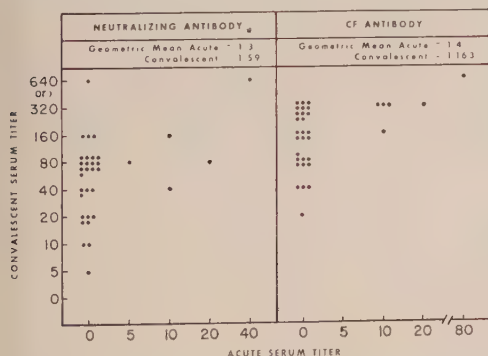


FIG. 1. Diagnostic neutralization and CF test findings in paired sera from 34 cases of respiratory illness caused by the respiratory syncytial virus.

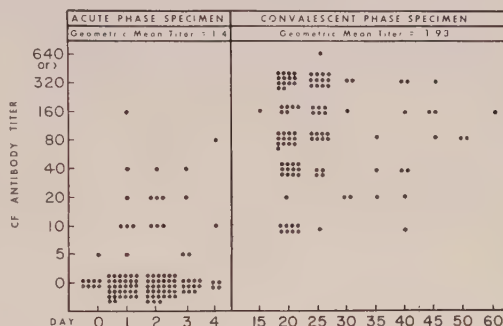


FIG. 2. Distribution, according to days post onset of illness, of CF titers in 109 cases of respiratory illness caused by the respiratory syncytial virus.

tory syncytial virus. These 34 cases were specially selected to include the syndromes of acute upper respiratory illness, bronchitis, bronchiolitis and bronchopneumonia. A single case of croup occurred among the cases but was not included in Table I. The acute phase serum specimens collected within 4 days of onset of clinical illness were usually devoid of neutralizing or CF antibody (<1:5) although a few sera were positive initially with titers as great as 1:80. The convalescent serum specimens regularly showed a diagnostic (4-fold or >) increase in titer of antibody. The maximum fold-increase by neutralization was 256 or > and by CF was 128 or >. The geometric mean* acute and convalescent neutralizing antibody titers

* In computing the mean titers, a titer of < 1:5 was arbitrarily assumed to be 1:2.5.

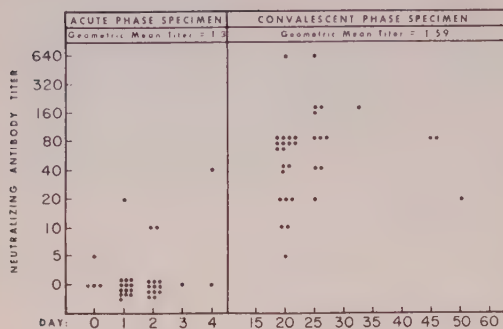


FIG. 3. Distribution, according to days post onset of illness, of neutralizing antibody titers in 34 cases of respiratory illness caused by the respiratory syncytial virus.

were 1:3 and 1:59, respectively, and mean CF titers were 1:4 and 1:163, giving mean fold-increases of 30 and 41, respectively. The serologic diagnoses usually could be confirmed by virus recovery.

Distribution of serum titers according to time post onset of illness. Fig. 2 summarizes acute and convalescent serum CF titers among 109 cases of respiratory illness diagnosed as respiratory syncytial virus infection based on the demonstration of a 4-fold or greater increase in CF antibody against the Long strain in tests with acute and convalescent sera. The geometric mean acute titer was 1:4 and mean convalescent titer was 1:93. The distribution of individual acute phase titers was roughly the same whether the specimens were collected on day 0, 1, 2, 3 or 4 post onset of illness. Maximal convalescent CF titers appeared to be reached by the fifteenth to twentieth day post onset of illness since titers of sera collected later were not appreciably different.

The findings in similar tests of neutralizing antibody in acute and convalescent sera from the 34 selected cases mentioned above are shown in Fig. 3. The geometric mean of the acute phase titers was 1:3 and of the convalescent was 1:59. In agreement with the CF results, the distribution of titer values in the acute phase specimens suggested no significant difference whether taken on day 0, 1, 2, 3 or 4 post onset of illness. Maximal neutralizing antibody titers appeared to be reached by day 20. It is of interest that a portion of the cases exhibited CF or neutra-

lizing antibody in the acute phase specimen, indicating that infection, with illness, apparently can be superimposed on antibody resulting from prior infection.

Virus recovery results. An attempt was made to recover respiratory syncytial virus from respiratory secretions of the 34 selected cases diagnosed, positively, by the CF method. The distribution of virus recoveries, according to time post onset of illness, is shown in Table II. Respiratory syncytial virus was

TABLE II. Distribution of Virus Recovery Results, According to Time Post Onset of Illness, among 34 Cases of Serologically Diagnosed Cases of Respiratory Illness Caused by Respiratory Syncytial Virus.

Result	Days post onset of illness specimen taken				
	0	1	2	3	4
Positive	3	9	8	0	1
Negative	1	6	4	1	1
% positive	(75)	60	67	(0)	(50)

recovered from 21 of the 34 cases (62%). Recoveries were made on days 0-4 post onset of illness indicating that the virus is present for at least 4 days after appearance of clinical symptoms. The recovered isolates were identified as respiratory syncytial agent both by CF and by neutralization tests. In all instances, there was complete agreement in the results.

Comparative sensitivity of diagnostic test methods. Table III summarizes results of diagnostic tests for 34 cases in which all 3 test methods were used. All 34 cases were diagnosed by CF and this result was confirmed by neutralization test findings in 33 cases (97%).

Respiratory syncytial virus was recovered from 21 of the 34 cases (62%). While the

TABLE III. Comparative Sensitivity of Various Diagnostic Test Methods for Detecting Respiratory Syncytial Virus Infections (Diagnosed Initially by CF).

Diagnostic test method	No. pos.	No. neg.	% positive
CF	34	0	100
Serum neut.	33	1	97
Virus recovery	21	13	62

virus recovery technic was the least sensitive of the 3 diagnostic tests, the number obtained is nonetheless an excellent experience for virus isolation results in general. It is important to note that 20 of the 21 virus recoveries were made in primary passage and that only 1 additional isolation was made by the routine subculture procedure. Further, only 1 or 2 of 3 inoculated tubes showed cytopathic effects on primary passage of some of the respiratory secretions.

Multiple serologic increases in antibody. Among the 563 respiratory disease cases studied, 19 additional persons showed a diagnostic CF titer rise against the respiratory syncytial virus plus adenovirus, Myxovirus parainfluenza 1, 2 or 3 or against influenza A₂. This indicated infection with more than 1 virus during the time period for the collection of the samples of blood. Adenovirus and parainfluenza 3 were encountered most frequently.

Discussion. These findings have clearly demonstrated the utility of both CF and serum neutralization tests for laboratory diagnosis of respiratory illness caused by the respiratory syncytial virus. Mean titers of the acute or the convalescent serum specimens were roughly the same when tested by the CF or serum neutralization technic and the two procedures appeared equally sensitive for detecting diagnostic increases in titer. These findings are at variance with those of Chanock *et al.*(2,3) who reported greater sensitivity of the neutralization technic, especially in infants and children in whom the neutralization test was stated to be twice as sensitive as the CF test. As a further test in our laboratory, paired sera from 13 cases of respiratory illness in very young infants 3 weeks to 5 months of age, collected at the height of a respiratory syncytial virus epidemic and found negative by CF, were tested by the neutralization method. The neutralization test results were also negative providing additional evidence that the CF test, properly performed, does not fail to detect cases.

The virus isolation test, while less sensitive than either of the 2 serologic procedures, was nonetheless highly efficient when appraised

in terms of expected recoveries in virus diseases, generally. Thus, the virus was recovered in 62% of 34 selected cases of the disease. Previous workers(1,2,3,5) have experienced great difficulty in isolating respiratory syncytial virus from respiratory specimens which had been frozen. These workers did not state whether the samples had been handled to exclude carbon dioxide. One worker used 2% horse serum in Hank's solution and another used Hank's solution alone for collecting and storing the specimens. These are probably inadequate for preserving infectivity. In the present series, the specimens had been stored frozen in veal infusion broth up to 1 year prior to testing. The data were insufficient to permit definitive appraisal of the relative efficiency of HeLa and human amnion cell cultures (WISH) for virus recovery. It was observed, however, in cultures inoculated with the same virus preparation, that the HeLa cells degenerated 1 or 2 days earlier than did the amnion cells.

The present findings, like those of Beem *et al.*(5), present convincing evidence for the role of respiratory syncytial virus in cases of human respiratory illnesses. Thus, the virus is present in the acute illness at time of the first visit to the physician and the patient develops high levels of both neutralizing and CF antibodies during convalescence. Additional support for etiologic role of this virus is presented in the second paper in this series(6) in which very low prevalence of infection is demonstrated among well controls.

The clinical illnesses which occur in infection with the respiratory syncytial virus include mild acute upper respiratory illness, bronchitis, bronchiolitis, bronchopneumonia and, infrequently, croup.

Summary. Laboratory diagnostic findings in 109 cases of respiratory illness caused by the respiratory syncytial virus are presented. The CF and serum neutralization tests were equally sensitive for detecting cases and antibody titers obtained by the 2 methods were roughly the same. The virus isolation procedure was less sensitive giving positive results in 62% of the cases. Clinical illnesses associated with respiratory syncytial virus infection included acute upper respiratory ill-

ness, bronchitis, bronchiolitis, bronchopneumonia and, rarely, croup.

The authors are indebted to B. Baron, P. Salvatore and I. Weinberger for technical assistance in the laboratory. Drs. K. C. Christie, P. J. Koblenzer, D. J. Bartels and A. Giriya assisted in clinical observations and in collection of specimens.

1. Morris, J. A. Blount, R. E., Jr., Savage, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1956, v92, 544.
2. Chanock, R., Roizman, B., Myers, R., *Am. J. Hyg.*, 1957, v66, 281.
3. Chanock, R., Finberg, L., *ibid.*, 1957, v66, 291.
4. Rowe, D. S., Michaels, R. H., *Pediatrics*, 1960,

v26, 623.

5. Beem, M., Wright, F. H., Hamre, D., Egerer, R., Oehme, M., *N. Eng. J. Med.*, 1960, v263, 523.
6. McClelland, L., Hilleman, M. R., Hamparian, V. V., Ketler, A., Reilly, C. M., Cornfeld, D., Stokes, J., Jr., *N. Eng. J. Med.*, in press.
7. Reilly, C. M., Stokes, J., Jr., McClelland, L., Cornfeld, D., Hamparian, V. V., Ketler, A., Hilleman, M. R., *New Eng. J. Med.*, in press.
8. Melnick, J. L., *Diagnostic Procedures for Virus and Rickettsial Diseases*, Am. Pub. Health Assn., N. Y., 2nd ed., 1956, 97.
9. Hayflick, L., *Exp. Cell Res.*, in press.

Received January 24, 1961. P.S.E.B.M., 1961, v106.

Resistance of Polyoma Virus Immune Animals to Transplanted Polyoma Tumors. (26453)

KARL HABEL

U. S. Department of HEW, National Institutes of Health, Nat. Inst. of Allergy and Infectious Diseases, Laboratory of Biology of Viruses, Bethesda, Md.

Previous reports(1,2), described establishment of transplantable lines of subcutaneous fibrosarcomas from tumors produced by inoculation of newborn animals with polyoma virus. Transplantable tumors in the hamster line and in one of the 2 mouse lines (C57-695) have never had demonstrable virus associated with them. The second mouse line (C57-1923) had virus associated with tumor for the first 10 transplant passages, but not in the subsequent 8 passages. Virus-negative tumors in tissue culture were susceptible to challenge with polyoma virus, and virus production could not be induced in them by irradiation. From this and the lack of demonstrable polyoma virus antigens in tumors, it was concluded that once virus had transformed normal cells to tumor cells it was no longer necessary for maintenance of the tumor.

As an additional piece of evidence for the above conclusion in studies of the hamster tumor(1), it was shown that adult hamsters made immune to virus and having demonstrable antiviral antibodies were still capable of supporting the transplantable tumor. However, this result was based on challenge with a large inoculum of minced tumor. The

purpose of this report is to present evidence indicating that adult hamsters and mice made immune to polyoma virus do develop a resistance to transplantable tumors provided the challenge with tumor cells is made on a quantitative basis. Evidence is also presented that this resistance is specific and not due to serum antibodies.

Materials and methods. Animals. General purpose (G.P.) mice were random-bred Swiss, C₃H were C₃H/Hen and C57 were C57/Bl. Hamsters were random-bred Syrian hamsters. All animals were reared in the NIH animal colony. *Tumors.* The transplantable polyoma hamster tumor and C57-695 and C57-1923 transplantable polyoma mouse tumors have been described(1,2). The SW tumor was a fibrosarcoma originating in a C57/Bl mouse that had been inoculated with radioactive thorium and was kindly supplied by Dr. Richard Swarm of Nat. Cancer Inst. in its 16th transplant passage. The S180 tumor was maintained in the ascites form in G.P. mice and was originally obtained from Dr. Morris Belkin of the Cancer Institute. *Virus.* Polyoma virus grown in mouse embryo tissue culture had a titer of 10⁷ ID₅₀/ml. *Hyperimmune rabbit serum.* Rabbits

TABLE I. Adult Hamsters Immunized with Polyoma Virus and Challenged with Hamster Tumor Cells 5 Weeks Later.

Cells transplanted	Normal hamsters		Immune hamsters	
	Tumors	Tumor size*	Tumors	Tumor size*
2.5×10^6	2/2	50×60 35×40	2/2	15×15 25×50
5×10^6	2/2	45×45 35×65	1/2	30×30
1×10^5	2/2	35×50 50×70	1/2	25×25
2×10^4	1/1	30×40	2/2	3×3 20×20

* Size of tumor in mm at 1 mo.

were immunized by an intramuscular dose of polyoma virus mixed with Freund's incomplete adjuvant and 8 intradermal doses in saline. It had a hemagglutinin inhibition (HI) titer of 1/10,000 and a neutralization titer of 1/25 against 100 TCID₅₀ of virus. *Immunization.* Mice referred to as "polyoma immune" had received 0.2 ml intraperitoneally (IP) of a 10^{-2} dilution of standard virus 4 to 10 weeks prior to a test. *Transplantation.* Tumors were maintained by subcutaneous (SC) inoculation of a heavy mince of tumor but quantitative tests used single cell suspensions of trypsinized tumors containing the indicated numbers of viable cells. All transplants were made into the interscapular subcutaneous area.

Results. Immunity to hamster tumor. Adult hamsters were inoculated IP with 0.5 ml of standard polyoma virus. Five weeks later the 24th transplant passage tumor was trypsinized and a single cell suspension counted. Groups of virus-immunized and control hamsters were inoculated SC with 0.1 ml of dilutions of this cell suspension (Table I). At time of tumor cell inoculation the immunized hamsters had polyoma HI anti-

bodies to a titer of 1/1600 in their serum. Animals were held for 2 months at which time 2 in the immune group still had no evidence of tumors, whereas all the controls were dead, with large tumors. In Table I and Fig. 1 can be seen the marked delay in tumor development in those virus-immune animals which did get tumors. *Immunity to mouse tumors.* Adult C57 mice were immunized by a single IP dose of 0.2 ml of 10^{-2} dilution of standard polyoma virus. Three weeks later they and a similar group of unimmunized mice received a heavy mince of transplant passage 12 of the 1923 tumor. After 4 months' observation, 5 of 5 normal mice had large tumors, whereas only 1 of 5 virus-immune mice had a much smaller tumor. A repetition of this experiment produced 2 of 5 controls with tumors and 0 of 5 immunes. (This tumor line has been difficult to transplant successfully in recent passages.)



FIG. 1. Normal hamster (left) and polyoma immunized (right) challenged with 2.5×10^6 hamster tumor cells 34 days before photograph.

TABLE II. Adult C57 Mice Immunized with Polyoma Virus and Challenged with 695 Tumor Cells 4 Weeks Later.

Cells transplanted	Exp. 1		Exp. 2	
	Normal	Immune	Normal	Immune
10^6	5/5*	5/5	5/5	3/5
10^5	5/5	2/5	5/5	0/5
10^4	3/5	0/5	0/5	0/5

* No. of mice developing tumors/No. inoculated.

With the 695 tumor, it was possible to quantitate susceptibility to the transplanted tumor. C57 mice were immunized as above and challenged 4 weeks later with 20th passage of the trypsinized 695 tumor. Table II shows that it required 10 times as many cells to produce a successful transplant in virus-immune mice as in controls. The same results were obtained in a repetition of this experiment (Table II). *Specificity of resistance.* To determine whether the demonstrated resistance produced by virus immunity was specific for tumor originally produced by polyoma virus or was a broad resistance to all

TABLE III. Adult Mice Immunized with Polyoma Virus and Challenged 6 Weeks Later with Indicated Tumor Cells.

Cells transplanted	S180 tumor		SW tumor	
	Normal	Immune	Normal	Immune
10 ⁶	5/5*	5/5	5/5	5/5
10 ⁵	4/5	3/5	5/5	5/5
10 ⁴	0/5	1/5	1/5	1/5

* No. of mice developing tumor/No. inoculated.

transplantable fibrosarcomas, 2 non-polyoma tumors were used. C₃H mice were immunized in the usual way with virus and challenged subcutaneously by serial dilutions of trypsinized S180 tumor cells obtained from an ascites passage of this tumor in GP mice. In a second experiment the same was done with dilutions of the SW tumor in C57 mice. Results given in Table III show equal susceptibility to these tumors by virus-immune and normal mice. *Effect of anti-polyoma serum antibody.* Groups of C57 mice were inoculated with serial dilutions of trypsinized 23rd passage of 695 tumor cells. One set of mice received 0.2 ml IP of polyoma hyperimmune rabbit serum, one set normal rabbit serum and one set no serum. The serum inoculation was repeated at 2 day intervals for 3 doses. Table IV shows no protective effect with the antiviral serum. *Anti-tumor complement fixation and cytotoxic antibodies.* Using serum from mice and hamsters made immune to virus and from virus-immune animals that had resisted transplantation of tumors, complement fixation and cytotoxic tests were run against the hamster and 695 mouse tumor. The complement fixation test was carried out with 2 full units of complement and overnight 4°C fixation. The cytotoxic test involved fully grown tube cultures of the tumor cells to which was added the serum to

TABLE IV. Serum Protection Test. C57 mice received 0.2 ml IP of normal or polyoma immune rabbit serum on day 1, 3 and 5. Day 1 indicated number of 695 tumor cells subcutaneously.

Cells transplanted	Normal serum	Immune serum	No serum
10 ⁶	5/5*	5/5	5/5
10 ⁵	5/5	5/5	5/5
10 ⁴	2/5	5/5	4/5

* No. of mice developing tumors/No. inoculated.

be tested, either alone or with fresh guinea pig serum, and observation for clumping or lysis of the tissue culture cells on incubation. With neither type of serum was there any evidence of complement-fixing or cytotoxic antibodies against the tumors.

Discussion and Summary. By producing an inapparent infection in adult hamsters or C57 mice with a single dose of diluted polyoma virus these animals subsequently manifest a relative resistance to transplantation of isologous polyoma fibrosarcomas. These sarcomas, although originally induced by virus inoculated into newborn animals, no longer contain any demonstrable virus or viral antigens. This resistance can be overcome if a large enough number of tumor cells are given in the challenge transplant; it is apparently specific for tumors of polyoma origin and would appear to be cell mediated since hyperimmune polyoma antiserum is not capable of transferring such resistance to normal mice.

The most logical explanation of this phenomenon is based on the hypothesis that polyoma induced tumors contain a cell antigen which is not present in normal isologous cells and the degree of this antigenic difference must be small. This new antigen would be produced by the normal cell after it has been transformed as the result of polyoma virus infection, both in the infected newborn and adult animal. The newborn being immunologically non-reactive would tolerate the foreign antigen and allow the transformed cells to multiply and produce a tumor. The adult animal being immunologically mature would reject the transformed cell with its foreign antigen and in so doing become hyper-reactive or resistant to the tumor when challenged with the transplant. This hypothesis is consistent with the findings of Law and Dawe(3) that adult mice given polyoma virus after whole body X-radiation do develop tumors.

If this hypothesis can be proven by immunological experiments, it would suggest that the genome of the virus-transformed cell either now contains viral genome or has been genetically altered by a non-lytic virus infec-

tion. In either case the result of this genotypic alteration would be the development in the transformed cell of an antigen immunologically slightly different from the corresponding antigen in the normal cell.

AND MED., 1959, v102, 99.

2. Habel, K., Silverberg, R., *Virology*, 1960, v12, 463.

3. Law, L. W., Dawe, C. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v105, 414.

1. Habel, K., Atanasiu, P., *PROC. SOC. EXP. BIOL.*

Received January 26, 1961. P.S.E.B.M., 1961, v106.

An Erythematous Disease of Adult Guinea Pigs Following Transplantation of Homologous Lymphoid Cells.* (26454)

JACK R. BATTISTO (Introduced by Edward J. Hehre)

*Department of Microbiology and Immunology, Albert Einstein College of Medicine,
Yeshiva University, New York City*

During studies on the transfer of delayed hypersensitivity to simple chemicals from highly sensitized to normal adult guinea pigs (1), occasional recipients of lymphoid cells developed an illness characterized by intermittent fever, squealing on handling, progressive weight loss, and generalized erythema. Evidence has now been obtained that this disorder is entirely independent of any artificial sensitization of the donor and may be produced with unprecedented high frequency by lymphoid tissue from a specific category of normal animals.

Following the discovery that certain guinea pigs possess natural, delayed type iso-hypersensitivity toward a heritable factor present in sera of other guinea pigs (2), an investigation was undertaken to determine whether a relationship might exist between this state of iso-hypersensitiveness and the illness following lymphoid cell transfer. As the following experiments show, the lymphoid tissue from guinea pigs lacking serum factor but possessing delayed iso-hypersensitivity toward it regularly induced the illness in adult recipients, while lymphoid tissue from guinea pigs possessing serum factor but lacking iso-hypersensitivity was incapable of inducing the disease.

The experimentally produced illness, which superficially resembles "runt", "homologous"

and "secondary disease" (3-7), but which differs from them by its induction in normal non-inbred adults and by its characteristic erythema, has been termed "erythematous disease" (8,9). Certain features of erythematous disease recall and allow comparison with aspects of "parabiotic intoxication" or "parabiotic disease" (10,11).

Methods. Experiments concerned with inducing erythematous disease consisted of transplanting washed lymphoid cells from one or more donor guinea pigs into one or more recipients after testing each animal for serum factor and for iso-hypersensitivity to the factor.[†] Guinea pigs bred from Rockefeller Institute stock, weighing more than 400 g and kept in temperature controlled quarters, were used throughout. Presence or absence of serum factor was determined by injecting 0.1 ml of each animal's serum intracutaneously into separate "assay" guinea pigs of known dermal reactivity (2); an erythematous response greater than 10 mm in diameter at 24 hours was deemed positive for serum factor. Possession or lack of iso-hypersensitivity was ascertained on day of transplantation by observing an animal's cutaneous reaction to sera, known to contain serum factor (2), inoculated intradermally

* This work was supported by grants from the Brown-Hazen Fund of Research Corp., and from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

[†] Such tests, done to provide information for the matching of animals, are not necessary for induction of erythematous disease as the illness can be produced in animals that have not received any prior injection.

TABLE II. Rejection of Skin Homografts in Normal Adult Guinea Pigs Possessing or Lacking Delayed Type Iso-hypersensitivity (IH) and Serum Factor (SF).

Donor		Recipient		No. grafts	Graft survival (days)
IH	SF	IH	SF		
+	—	—	+	4	13
+	—	+	—	11	13-15
—	+	—	+	22	13-17
—	+	+	—	4	9-14

This mechanism might conceivably account for the high incidence of the disease in recipients with serum factor (Exp. A); however, it could not account for the equally high incidence of the disease observed in recipients lacking serum factor and possessing iso-hypersensitivity (Exp. B; Fig. 1). An alternative basis for a "graft-versus-host" mechanism would be provided if the effective lymphoid cells (which are from animals genetically lacking serum factor) were for some reason selectively tolerated by the recipient hosts. However, in an experiment in which full thickness (1 cm²) skin grafts were exchanged among guinea pigs of Rockefeller Institute stock, matched for possession or lack of serum factor and iso-hypersensitivity, no immunological tolerance to homologous tissue was detected (Table II). Thus, all skin homografts were rejected within a time range (9-17 days) not far removed from that which others have noted for first-set homografts (12); whereas, a majority of autologous (control) grafts appeared well seated and healthy at 22 days and beyond.

The possibility that some mechanism other than graft-versus-host reaction may be re-

sponsible for erythematous disease is being explored.

Summary. Adult, immunologically competent, non-inbred guinea pigs injected with selected homologous lymphoid cells develop a wasting, sometimes fatal illness. Termed "erythematous disease," it is induced by spleen and lymph node cells from guinea pigs lacking heritable serum factor toward which they possess naturally occurring delayed type iso-hypersensitivity.

The author wishes to thank Dr. Merrill W. Chase for making guinea pigs of the Rockefeller Inst. stock available for breeding; Dr. Irwin M. Dannis for conducting the skin graft experiment; Miss Janet F. Parks and Miss Rita Hixon for capable technical assistance.

1. Battisto, J. R., Chase, M. W., *Fed. Proc.*, 1955, v14, 456.
2. Battisto, J. R., *Nature*, 1960, v187, 69.
3. Billingham, R. W., Brent, L., *Transpl. Bull.*, 1957, v4, 67.
4. Cole, L. J., Ellis, M. E., *Science*, 1958, v128, 32.
5. Simonsen, M., *Acta Path. Microbiol. Scand.*, 1957, v40, 480.
6. Trentin, J. J., *Fed. Proc.*, 1958, v17, 461.
7. Schwartz, E. E., Upton, A. C., Congdon, C. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1957, v96, 797.
8. Battisto, J. R., *Bact. Proc.*, 1960, 86.
9. ———, *Ann. N. Y. Acad. Sci.*, 1960, v87, 468.
10. Herrmannsdorfer, A., *Deutsch Z. für Chir.*, 1923, v178, 289.
11. Nakic, B., Silobrcic, V., *Nature*, 1958, v182, 264.
12. Bauer, J. A., Jr., *Ann. N. Y. Acad. Sci.*, 1958, v73, 663.

Received January 27, 1961. P.S.E.B.M., 1961, v106.

A Simple, Rapid Skin-Graft Technic in Rats. (26455)

ARTHUR N. THOMAS, DONALD L. MORTON AND RICHARD E. GARDNER

Surgical Research Laboratories, Department of Surgery, University of California School of Medicine, San Francisco

A modification of the punch technic for skin grafting as performed on mice(1,2) has been successfully adapted to rats. Improvements developed during studies on 242 rats have resulted in a regularly obtained healthy

skin graft. Sutures are unnecessary in this technic. In mice a tape vest can be used(3), but because of the greater strength of rats, we have found it necessary to use a plaster jacket. The graft is well immobilized in a



FIG. 1. Punch skin graft technic in rats. A. A double layer of skin is elevated in preparation for the punch graft. B. Stratum carnosum is removed from each of the 2 skin discs. C. Skin is placed in graft bed and immobilized with a second strip of tape and covered with a gauze roll. D. Position of the plaster cast at completion of graft. E. Case and dressing are removed in 8 days. A superficial layer of skin is removed with the tape, leaving viable skin graft.

perfectly fitted graft bed by using light vertical pressure.

Method. Young adult male Long-Evans rats, weighing 100 to 150 g, were anesthetized by ether inhalation. "Scotch" Brand pressure-sensitive tape (2.5 cm wide) was used in all animals. A leather punch, with a blade 1.25 cm in diameter, was made to cut against a nylon pad, to avoid dulling the blade's cutting edge (Fig. 1-A). Sponge rubber in the lumen of the blade eliminated the need to remove the graft manually, which often disturbs the graft's adherence to the tape. The graft site was on the posterior chest wall, 2.5 cm caudad to the shoulder girdle. This area could be well covered by the plaster cast without interfering with the shoulder girdle. Grafts placed cephalad to this site were often inadequately immobilized because of neck movement.

The hair of the graft area is shaved and the area is prepared with tincture of iodine. After 3 minutes the area is washed with ether to remove moisture and skin oils. A 5 cm strip of "Scotch" tape is pressed onto the skin. The tape and adherent skin are elevated carefully by pinching the tape in the center (Fig. 1-A). The elevated tape with 2 layers of skin, panniculus adiposus and panniculus carnosus are punched as one. The 2 discs that are obtained are connected by a

fibrous band, which is then divided. The panniculus carnosus is then excised with scissors; care is taken not to separate the skin from the adherent tape (Fig. 1-B). The muscle layer is excised most easily if the scissor blades are held parallel to the direction of the muscle fibers.

The graft bed requires no preparation, and bleeding is negligible. The circular disc is inserted into the same-sized defect, with the surrounding tape in place (Fig. 1-C). The graft must be well immobilized to insure that a "take" will occur. A second 5 cm strip of tape is placed over the discs and the first strip of tape. The double thickness of tape is then covered by a doubled 5×5 cm layer of gauze and is ready for casting. A single sheet of plaster splint, measuring 7.6×38 cm, is folded at one-third its width and is wetted. Warm water is used for more rapid setting of the plaster. The cephalad and caudad edges of the plaster are made slightly constrictive by winding the splint obliquely (Fig. 1-D). If the fit is too loose, the rat can wriggle out of the plaster. Picric acid can be used to prevent the rats from chewing the case, but this is seldom necessary.

The ventral aspect of the cast is cut with heavy scissors on the eighth day. The plane between the 5×5 cm gauze and the tape is found so that the tape can be removed separately from the plaster. The end of the tape is slowly lifted up, with gentle separation of the tape from the skin. A scalpel blade can be used to apply counter-traction and prevent pulling off the graft. A plane of cleavage appears in the disc of rat skin graft (Fig. 1-E) between the stratum corneum and the stratum lucidum. The implanted graft disc of skin without hair or outer dessicated squamous cells is smooth and glistening.

Results. The early detection of onset of the homograft reaction, with the graft denuded of hair and dessicated cells, is remarkably easy. Very minor amounts of edema and changes in the color or size of the graft can be readily identified. The later stages of the homograft reaction are obvious.

Summary and conclusions. A modified graft technic is described which has been found to be rapid and reliable in rats. Two

people can complete this graft technic in 15 to 25 rats in one hour. The adherence of the skin, hair and outer dessicated layers of the skin to the tape allows an unobstructed view of the viable graft and permits detection of minor changes, as compared with the control site. When the graft disc was placed without an overlying tape, or when the cast and tape were removed by the animal, the superficial dessication and hair prevented the early changes of slight edema, faint brown-

ness in color, and reduction of graft diameter, from being seen or recognized as the early onset of the homograft reaction.

1. Billingham, R. E., Medawar, P. B., *J. Exp. Biol.*, 1951, v28, 385.
2. Gottfried, B., Padnos, M., *Transpl. Bull.*, 1959, v6, 427.
3. Gross, L., Padnos, M., Gottfried, B., *ibid.*, 1960, v25, 421.

Received February 1, 1961. P.S.E.B.M., 1961, v106.

Fluorescent Treponemal Antibody Test Using the Reiter Treponeme.* (26456)

SCOTT V. COVERT, JOHN F. KENT AND ROY W. STEVENS

*Albany Medical College and Division of Laboratories and Research, New York State
Dept. of Health, Albany*

The fluorescent treponemal antibody (FTA) test(1,2) for syphilis requires *Treponema pallidum* of the virulent Nichols strain as antigen, and as a result is expensive and limited in availability. This suggested an attempt to substitute the Reiter strain of treponeme, which is antigenically related and easily cultivated *in vitro*.

Materials and methods. Sera were from a collection of the *T. pallidum* immobilization (TPI) testing unit of the Division of Laboratories and Research; they were from persons verified as syphilitic or non-syphilitic on the basis of clinical or anamnestic evidence, supplemented by a critical test(3,4) for TPI antibody; many of the non-syphilitic individuals presented diagnostic problems in that they reacted serologically with cardiolipin antigens. Normal sera were from healthy members of both staffs and from blood donors. All sera were stored at -25°C . *T. pallidum* of the Reiter strain was cultivated at 35°C in screw-capped tubes con-

taining 25 ml NIH thioglycollate broth[†] to which heated (2 hours at 56°C) rabbit serum had been added to give a final concentration of 10%. Growth was satisfactory at 2 days, the cultures remaining acceptable for antigen preparation from the 2nd to 5th day. Two ml of culture were removed aseptically from the undisturbed upper stratum and delivered into a 15×100 mm pyrex tube containing 2 ml 0.002% sodium hypochlorite (NaClO)[‡] in 0.85% NaCl solution. The NaClO was essential, the treponemes otherwise clumping, becoming atypical in morphology, and failing to adhere to slides. They were mixed well by gentle swirling, then sedimented by spinning 15 minutes at 1,000 g. The supernate was decanted, and the organisms were washed once by resuspending in 4 ml 0.85% NaCl solution, centrifuging, and decanting as before. They were suspended finally in enough 0.85% NaCl solution to make the count 10-15/430 \times microscopic field, *i.e.*, about $2 \times 10^6/\text{ml}$. *T. pallidum* of the Nichols strain was available as suspensions eluted from fresh or frozen(5) syphilomatous rab-

* Aided in part by a grant to Albany Medical College from Sterling-Winthrop Research Inst., Rensselaer, N. Y. Special thanks go to Herbert W. Reilly, Jr., and Frederick W. Bauer, Jr., who assisted with the RFTA and FTA tests, and to Jacob B. DeWeerd and Janet G. Jennings who provided the Nichols strain treponemes.

[†] No. 0257-01, Difco Labs., Inc., Detroit, Mich.

[‡] B.K. chlorine-bearing liquid bactericide; active ingredient sodium hypochlorite 5.25%. Pensalt Chemicals Corp., Phila. 2, Pa. A 1:2,625 dilution was made in 0.85% NaCl solution.

bit testes in Nelson's medium(6) containing streptomycin and penicillinase(7); they were used on the day of elution or after 1-15 days' storage at 2-4°C. For FTA tests they were adjusted with Nelson's medium to contain 40-50 treponemes/430 \times microscopic field, a count 3 to 4 times that of the Reiter suspension. This compensated for reductions in number/field that occurred during treatment of the films with serum and fluorescent antibody; ordinarily 10-15/field remained for final examination. No such losses occurred with the NaClO-treated Reiter treponemes. New precleaned microscope slides (75 \times 25 \times 1 mm) and cover slips (22 \times 22 mm, thinness No. 1) were used: two 1-cm circles were inscribed on the former with a template and diamond stylus. Slides and cover slips were soaked 1 hour in 95% ethanol, then wiped smooth with clean gauze followed by disposable tissue.[§] They were stored in dust-free containers. The technic of fluorescent antibody testing was essentially that given (1,2) for the FTA test. Aliquants (0.01 ml) of an antigen suspension were delivered into each of the circles on a slide and spread to fill the circles with a glass rod. The films were air-dried, after which the slides were immersed 10 minutes in acetone (Certified Reagent), and again air-dried. Sera to be tested were heated 30 minutes at 56°C, and a 0.1-ml sample diluted 1:200 stepwise by adding 1.9 ml phosphate buffered saline solution of pH 7.2 (PBS),^{||} mixing well, transferring 0.10 ml to a tube containing 0.9 ml PBS, and again mixing well. The 1:200 dilution sufficed for qualitative tests; for quantitative testing, further serial 2-fold dilutions were made in PBS. In the test proper, 0.03-ml aliquants of diluted serum were added to 2 films each of the Reiter and Nichols tre-

ponemes, and the slides rotated[¶] 30 minutes at 100 rpm in the incubator (37°C); they were protected from evaporation by lids containing moistened blotting paper. The slides were then rinsed in PBS, soaked for 5 minutes in each of 2 changes of PBS, and pressed gently on filter paper to remove excess moisture. Fluorescein-labeled horse antihuman globulin** diluted optimally (1:30) in PBS containing 2% Tween 80^{††} was then added, 0.03 ml being distributed over each film, and the slides again rotated 30 minutes at 100 rpm in the incubator. They were washed twice in PBS as before and blotted gently on filter paper. Finally, a drop of buffered glycerol (9 parts glycerol + 1 part PBS) was placed on each reaction site and surmounted with a cover slip. Slides were examined by darkfield microscope,^{‡‡} using low fluorescence immersion oil^{§§} between condenser and slide. Fields containing treponemes were located first by visible light, and the treponemes then examined for fluorescence by ultraviolet light. Degree of fluorescence was estimated, and the sera designated reactive (2+ to 4+ fluorescence) or non-reactive (- to 1+). Reactive specimens were diluted serially in PBS and retested. Reactivity restricted to the 1:200 dilution was represented as a titer of 1; reactivity extending to higher dilutions (1:400, 1:800, etc.) was represented similarly as 1/200th the reciprocal of the highest reactive dilution (titer = 2, 4, etc.).

¶ Rotating apparatus, electric, Cat. No. 3623, A. H. Thomas Co., Phila., Pa.

** Sylvana Chemical Co., Orange, N. J.

†† Hill Top Laboratories, Inc., Cincinnati, Ohio.

‡‡ American Optical Co., Model 2; objective, achromatic, 43X; condenser, C234, wide angle, with darkfield stop plate. Illumination, Reichert "Fluorex" with power supply. Subocular filter OG-1 used continuously to exclude visible blue and residual UV light; filter BG-12 interposed for the switch from unrestricted to UV illumination. Intensity of illumination controlled by Weston Master III universal exposure meter inverted on window of Reichert illuminator; adjustment to meter reading "800" made with illuminator diaphragm.

§§ Type A, R. P. Cargille Laboratories, Inc., New York, N. Y.

§ Kimwipes, Kimberly-Clark Corp., Neenah, Wis.,

|| One liter of aqueous solution contained 6.8 g NaCl, 1.48 g Na₂HPO₄, and 0.43 g KH₂PO₄. The formula differed slightly from that prescribed for FTA tests (Bacto Hemagglutination Buffer No. 0512, Difco Labs., Inc., Detroit, Mich.), but had better buffering capacity and did not affect FTA results. It was substituted primarily because it was regularly available from laboratory stocks(8).

TABLE I. Fluorescent Treponemal Antibody (FTA) Tests of 51 Syphilitic Persons Using Reiter (R) and Nichols (N) Strains of *Treponema pallidum*.

		Numbers of persons yielding the given titers					
FTA(R) titer	16					1	
	8					3	
	4		4	3	1		
	2		7	10			
	1	4	14	3			
	<1		1				
		<1	1	2	4	8	16
		FTA(N) titer					

Results and discussion. The relative sensitivities of the RFTA (Reiter Fluorescent Treponemal Antibody) and FTA tests were determined by parallel titration of syphilitic sera; aliquants of the same serum dilutions served for both tests. RFTA and FTA titers of 51 individuals are represented in Table I. They were the same in most instances. However, the RFTA titer was twice the FTA in 16 instances, and one-half the FTA in 5 instances. To determine whether a significant difference existed, RFTA and FTA titers were transformed to logarithms and the differences between the logarithms calculated. An analysis(9) of these differences, based on Student's *t*, yielded a value of 2.53, which would be expected less than 2% of the time if the tests were the same. The RFTA test thus appeared slightly but significantly more sensitive for syphilitic antibody than the FTA test. Relative specificity for syphilis was evaluated by testing qualitatively sera from 1) 103 healthy individuals, and 2) 38 persons with non-treponemal infections or non-infectious disorders who were negative in a critical 40-hour test(3,4) for TPI antibody; the

majority (35) of the latter reacted in standard tests with cardiolipin antigens, and so provided a more critical test of specificity than sero-negative individuals. None of the healthy persons yielded RFTA or FTA reactions. One of the "non-specific" seroreactors reacted (titer = 1) in the RFTA test but was negative in the FTA. The test using Reiter treponemes requires further evaluation. However, present indications are that it may substitute effectively for the FTA test. The possibility of substituting an organism simply cultivated *in vitro* for one requiring *in vivo* maintenance holds advantages in simplicity and economy for the physician and diagnostic laboratory.

Summary. A fluorescence test for treponemal antibody using the *in vitro* cultured Reiter treponeme compares favorably in sensitivity and specificity for syphilis with one requiring the virulent Nichols strain of *T. pallidum* which must be cultivated *in vivo*.

1. Deacon, W. E., Falcone, V. H., Harris, A., *Proc. Soc. Exp. Biol. and Med.*, 1957, v96, 477.
2. Deacon, W. E., Freeman, E. M., Harris, A., *ibid.*, 1960, v103, 827.
3. Rosenau, B. J., Kent, J. F., *J. Lab. and Clin. Med.*, 1958, v51, 664.
4. Kent, J. F., DeWeerd, J. B., *Am. J. Clin. Path.*, 1961, 35, in press.
5. Anderson, R. I., Kent, J. F., Sanders, R. W., *Am. J. Syph., Gonorr., and Ven. Dis.*, 1954, v38, 527.
6. Nelson, R. A., Jr., Diesendruck, J. A., *J. Immunol.*, 1951, v66, 667.
7. Borel, L. J., Bentejac, R., Durel, P., *Brit. J. Ven. Dis.*, 1958, v34, 241.
8. Wadsworth, A., *Standard Methods of Division of Laboratories and Research of New York State Department of Health*, 3rd edition, Williams and Wilkins Co., Baltimore, 1947, 226 (Formula 68.5).
9. Brownlee, K. A., *Industrial Experimentation*, Chemical Publishing Co., Inc., Brooklyn, 1949, 32.

Received February 2, 1961. P.S.E.B.M., 1961, v106.

Interarterial Coronary Anastomoses in Neonatal Pigs.* (26457)

LEOPOLD REINER, DOBRILA VRBANOVIC AND ALFONSO MADRAZO
(Introduced by Alfred J. Weil)

*Department of Laboratories, The Bronx Hospital, and Department of Pathology,
Albert Einstein College of Medicine, New York City*

The hearts of human neonates born at and beyond term, are endowed with interarterial coronary anastomoses in a high percentage of cases, as demonstrated by a technic of post-mortem arteriography based upon unilateral injection of the right or left coronary artery with a radiopaque mass(1). With this technic it was disclosed that interarterial coronary anastomoses are not to be viewed as an all-or-none phenomenon but rather as a spectrum of vascular communications which could be graded on a scale of from 0 to 4+ depending on the arteriographic richness of vascular filling in the contralateral arborization. Collaterals of any grade were found in 4/5 of all cases; fully one-third exhibited 4+ collaterals. The question was raised whether these findings were characteristic of the neonatal state of man or whether they also applied to other mammals. To this end, the hearts of neonatal pigs were examined.

Materials and methods. Twenty-nine newborn and young suckling pigs were obtained from 3 different sources in Westchester and Dutchess Co., New York. Of these, 7 were stillborn having died presumably at or near term as judged by their birth weights; the remainder died within 2 hours after birth or were killed during the first 12 postnatal days by ether inhalation or by a blow on the head. In Table I the animals are listed according to sex, age and weight of body and heart. The 29 animals belonged to 17 litters, 4 of these litters being represented by 2, 2, 4 and 8 animals, respectively. All the animals were offspring of primiparous sows bred soon after attainment of sexual maturity at the age of 8-9 months and approximately 1 year of age at time of delivery. (Gestation in the pig according to the mnemonics of breeders takes 3 months + 3 weeks + 3 days).

Apart from atresia ani in a few of the ani-

mals no developmental abnormalities were noted. Two animals showed fibrous adhesions between loops of the small intestine.

The technic of arterial injection was identical with that employed in the study of human hearts(1). In short, it comprised the following steps: 1) Cannulation of right and left coronary arteries with polyethylene tubing; 2) Flushing of both coronary arteries with 1-2 cc of physiological (0.9%) saline solution by a hand-operated syringe; 3) Unilateral injection of either right (13 cases) or left (16 cases) coronary artery with a highly viscous, radiopaque mass (barium sulfate in gelatine at pH 6.2)(2) for 10 minutes and at a pressure which was gradually raised to 150 mm Hg; 4) "Unrolling" of the heart (3); 5) Stereoscopic x-ray photography.

Results. In all the hearts the arborization injected showed optimum filling as judged by the appearance on X-ray films. However, in none of the hearts was any injection mass seen in the contralateral arborization. The injection mass here employed advances quite regularly into vessels having a diameter of about 40 μ (2) and irregularly into still smaller vessels (as measured in paraffin sections after formalin fixation of the tissues), without entering capillaries or crossing over into veins. Thus, the findings indicate the absence of interarterial coronary anastomoses of about 40 μ or larger in the hearts of neonatal pigs, under the conditions of the experiments. These findings are essentially identical with those of a previous series in which the above technic was applied to hearts of 18 adult pigs of unknown sex and age. Of these 18 hearts, 17 showed no collateral flow and 1 showed 1+.

Discussion. Postmortem injection of hearts of human neonates previously reported has revealed the existence of interarterial coronary anastomoses in a high percentage of those born at or beyond term, in-

*Supported by Research Grant from Nat. Heart Inst., N.I.H.

TABLE I. Distribution of Neonatal Pigs by Sex, Age and Weights of Body and Heart.

Age	No. of cases	Sex		Wt (g) of	
		♂	♀	Body	Heart
<i>Stillborn</i>					
—	7	4	3	930-1170	7.5-10.5
<i>Liveborn</i>					
2 hr	10	6	4	590-1220	6.0- 8.5
1- 3 days	6	4	2	830-1360	7.5-12.5
4-12 "	6	4	2	2010-2940	15.0-35.0

cidence and degree of such collaterals increasing with weights of body, brain and heart. In analogy to the observations of Zoll *et al.* (4) in adults the possibility was considered that development of collaterals in term and overdue babies was mediated by intrauterine hypoxia including that due to deterioration of fetal oxygen supply which, according to Walker & Turnbull(5) occurs in late and especially in postmature stages of human pregnancy, as well as in pre-eclampsia and other abnormal states. Interarterial coronary anastomoses were observed also in premature neonates but, for undetermined reasons, only in those born of mothers 23 years of age or under. In some of these premature cases, development of collaterals may have been related to maternal toxemia; in some others, coexistent congenital heart disease in the neonate may have played a role.

The absence of interarterial coronary anastomoses in hearts of neonatal (term) pigs indicates that the factor or factors which in human neonates govern development of collaterals in general, and of high-grade collaterals (3+ and 4+), in particular, do not operate in the pig. The difference between the 2 species may be an organ characteristic since under natural conditions collaterals are absent not only in the newborn pig but with rare exceptions also in the adult animal whereas in normal hearts of man (low-grade) collaterals are a common finding throughout postnatal life. On the other hand, the difference between the 2 species may be secondary to specifics of the intrauterine environment including differences in degree and duration of hypoxia, in adaptation on the part of the fetus or in both. Since interarterial coronary anastomoses in human neo-

nates, especially those of high grade, might be looked upon as altogether pathological and the result of such states of pregnancy as are also associated with increased perinatal mortality, it is perhaps significant that no collaterals had developed even in the pig fetuses which had died either *in utero* or shortly after delivery.

Differences of placental structure(6) probably played no role since the hemochorial placenta of man would be expected, if anything, to provide a better oxygen supply to the fetus than the epithelial-chorial placenta of the pig. In the former, maternal blood circulates freely in the intervillous space and oxygen has to travel through only 3 anatomic layers (villous epithelium, villous stroma and endothelium of fetal capillaries) whereas in the latter, 6 layers intervene between maternal and fetal blood (endothelium, stroma, and epithelium on the maternal side and, in reverse order, on the fetal side). However, Dempsey(7) has pointed out that "the placental type does not accurately describe the distance across which metabolites must be transferred" and that it is therefore unjustified to draw physiological inference from anatomic schemes. Amoroso(8) reported that of all the placentas examined (by Louis Flexner) only the placenta of the pig failed to show a terminal decline of sodium transfer, that "it is the only one which remains functionally active to term and even seems to improve with age" and that it does not display the senescent changes seen in placentas of most other mammals. The possible relevance of these remarks to the current topic is obvious and calls for investigation of interarterial coronary anastomoses in other mammalian species.

Summary. The right or left coronary arteries of the hearts of neonatal pigs were injected with a highly viscous barium sulfate-gelatine mass which quite regularly fills vessels down to a diameter of about 40 μ and which fails to enter capillaries or cross over into veins. No anastomoses were demonstrated in 29 hearts examined. This is in contrast to the findings in hearts of human term neonates which are endowed with collaterals of varying grades in most cases. The

differences between the 2 species indicate that interarterial coronary anastomoses in hearts of neonates are not a general characteristic of the mammalian phylum but rather of man. Whether they are unique to the latter requires the study of neonatal hearts in other species.

1. Reiner, L., Molnar, J., Jimenez, F. A., Freudenthal, R. R., *Arch. Path.*, 1961, v71, 103.
2. Schlesinger, M. J., *Lab. Invest.*, 1957, v6, 1.
3. Rodriguez, F. L., Reiner, L., *A. M. A. Arch. Path.*, 1957, v63, 160.

4. Zoll, P. M., Wessler, S., Schlesinger, M. J., *Circulation*, 1951, v4, 797.
5. Walker, J., Turnbull, E. P. N., *Lancet*, 1953, v2, 312.
6. Arey, L. B., *Developmental Anatomy. A Text-book and Laboratory Manual of Embryology*. W. B. Saunders Co., Philadelphia and London, 1954.
7. Dempsey, E. W., In: *The Placenta and Fetal Membranes*. Edited by Claude A. Villee. Williams & Wilkins Co., 1960, p30.
8. Amoroso, E. C., *idem.*, p243.

Received February 6, 1961. P.S.E.B.M., 1961, v106.

Allergic Reactions to Hyaluronidase.* (26458)

LEON S. KIND AND SUZANNE ROFFLER

Department of Microbiology, University of California Medical Center, San Francisco

During the past several years a number of reports of allergic reactions to hyaluronidase have been published(1-5). Holborrow and Keech(1) believed that the occurrence of hypersensitivity reactions in their patients was probably due to presence of protein impurities in the hyaluronidase preparations employed. Although the purity of more recent preparations has been emphasized(2), the data to follow will indicate that a highly purified commercial hyaluronidase extracted from bull testes was found to contain considerable bovine serum proteins. Such impurities could easily have been responsible for the reported allergic reactions to hyaluronidase.

Experimental. An adult albino rabbit was injected subcutaneously once or twice weekly with 1-4 mg of hyaluronidase (Wydase)[†] in Freund's adjuvant. A total of 16 mg was administered over 5 weeks. Five days after the last injection the animal was bled and its serum tested for antibodies to bovine serum albumin (BSA) and bovine gamma globulin (BGG) by the hemagglutination technic of Stavitsky and Arquilla(6). BSA or BGG coupled to rabbit red blood cells with bis-diazotized benzidine served as antigens.

Table I shows that the serum of the rabbit injected with hyaluronidase had a titer of 1:10,240 to BSA and a titer of 1:10,240 to BGG. Pre-incubation of this serum with 1 mg of hyaluronidase reduced the titer to zero. In the next experiment a series of dilutions of a rabbit anti-BSA serum (titer 1:25,600) was incubated with various amounts of BSA or hyaluronidase prior to addition of BSA coupled to red cells. Table II demonstrates that 1 mg of hyaluronidase reduced the anti-BSA serum titer to zero and 0.1 mg lowered the titer to 3200; .01 mg of hyaluronidase had no inhibitory effect. Comparison of the above inhibition with that produced by BSA (Table II) suggests that the hyaluronidase preparation employed had 1/100 to 1/1000 the inhibiting activity of BSA. Pre-incubation of rabbit anti-rye grass serum with 1 mg of hyaluronidase did not lower the titer of this serum to rye grass pollen (Table II).

In a third experiment mice were sensitized to BSA by intraperitoneal injection of 5 billion *Bordetella pertussis* organisms and 4 mg of BSA(7). Ten days later the animals were challenged intravenously with various amounts of BSA or hyaluronidase. Due to the small amounts of material used in challenge, fatal anaphylaxis did not take place

* This investigation was aided by research grants from Nat. Inst. of Health, U.S.P.H.S.

[†] Wyeth Laboratories, Philadelphia, Pa.

TABLE I. Demonstration of Antibodies to Bovine Serum Proteins in Serum of a Rabbit Injected with Hyaluronidase.

Rabbit antiserum	Inhibiting antigen*	Antigen on red cell	Reciprocal of titer
Anti-hyaluronidase	—	BSA†	10,240
"	—	BGG‡	10,240
"	1 mg hyaluronidase	BSA	0

* Incubated with antiserum for 30 min. at room temperature prior to addition of antigen on red cell.

† Bovine serum albumin.

‡ Bovine gamma globulin.

(ordinarily 1 mg of BSA will kill 50-75% of the animals). The occurrence of anaphylaxis was determined by noting symptoms and recording the fall in rectal temperature after challenge(8,9). The data in Table III demonstrate that both BSA and hyaluronidase caused symptoms of anaphylaxis and a fall in rectal temperature in BSA sensitive mice. The activity of the hyaluronidase preparation was approximately 1/200 to 1/1000 that of the BSA, a ratio very similar to that obtained by the hemagglutination inhibition technic (Table II). There seems little doubt that our hyaluronidase preparation contained bovine serum albumin (0.1-1%), bovine gamma globulin, and other serum compon-

TABLE II. Inhibition of Hemagglutination by BSA and Hyaluronidase.

Rabbit antiserum	Inhibiting antigen	Antigen on red cell	Reciprocal of titer
Anti-BSA	—	BSA	25,600
"	1 mg hyaluronidase	"	0
"	.1 " "	"	3200
"	.01 " "	"	25,600
"	10 µg BSA	"	0
"	1 " "	"	400
"	.1 " "	"	3200
Anti-rye grass	—	Rye grass	25,600
Idem	1 mg hyaluronidase	Idem	25,600

ents, although it is conceivable that the results obtained were due to the fact that bovine hyaluronidase has antigenic determinants similar to those in BSA or BGG. In view of the probability that other commercial hyaluronidase preparations also contain bovine serum proteins as impurities, it may be advisable to skin test a patient with bovine serum prior to administration of hyaluronidase.

TABLE III. Anaphylaxis in BSA Sensitive Mice after Challenge with BSA or Hyaluronidase.

Mice sensitized with	Challenged 10 days later with	Reactors* /Total
—	25 µg BSA	0/10
.1 ml pertussis vaccine + 4 mg BSA	5 " "	1/8
Idem	25 " "	6/8
"	5 mg hyaluronidase	5/10
"	2.5 " "	0/7
—	5 " "	0/10

* Symptoms of anaphylaxis plus a temperature drop greater than 2°C.

Summary and conclusions. It has been demonstrated by hemagglutination inhibition tests as well as by induction of anaphylactic shock that a commercial hyaluronidase preparation contained a small but appreciable amount of bovine serum proteins. In view of the known allergenic properties of such impurities it is suggested that skin tests with bovine serum precede administration of hyaluronidase.

1. Holborrow, E. J., Keech, M. K., *Brit. Med. J.*, 1951, v2, 1173.
2. Kendall, H. P., *ibid.*, 1957, v1, 1419.
3. Barber, K., *ibid.*, 1957, v1, 166.
4. Joubert, P. J., *ibid.*, 1957, v1, 46.
5. Piercy, M. L., *ibid.*, 1960, v1, 1655.
6. Stavitsky, A. B., Arquilla, E. R., *Int. Arch. Allergy*, 1958, v13, 1.
7. Malkiel, S., Hargis, B. J., *Proc. Soc. Exp. Biol. and Med.*, 1952, v80, 122.
8. Kind, L. S., *J. Immunol.*, 1955, v74, 387.
9. ———, *Bacteriol. Rev.*, 1958, v22, 173.

Received February 6, 1961. P.S.E.B.M., 1961, v106.

Sensitivity of Various Viruses to Chloroform.* (26459)

HARRY A. FELDMAN AND STEPHEN S. WANG

Department of Preventive Medicine, State University of New York, Upstate Medical Center, Syracuse, N. Y.

Several authors(1,2) have suggested that identification of certain viruses could be simplified by determining their susceptibility to ether or sodium desoxycholate. Although such sensitivity appears to be a rather constant characteristic of some viruses, it appears to be a group attribute and thus should be of considerable utility in the initial classification of a newly isolated, unidentified agent. Of the 2 systems, that utilizing ether has perhaps been employed more often but with considerable variation in the procedures. These have been in terms of the ether and concentration employed and temperature and time of exposure. Finally, the residual ether must be removed by evaporation. Sodium desoxycholate has its own disadvantages in that solutions have to be prepared and sterilized for this purpose.

It occurred to us that if the method could be more readily standardized and simplified it could be of considerable aid, especially for those working in the respiratory virus field. Chloroform suggested itself as possibly a more efficient substitute for ether because of its greater polarity as a lipid solvent. It offered the additional advantage of being heavy and, therefore, readily separable by sedimentation. It was found that those agents that are susceptible to ether are also susceptible to chloroform while those that are resistant to ether are not affected by chloroform.

Materials and methods. *Chloroform.* This was of the analytical reagent grade (Mallin-krodt). *Viruses.* The various strains employed were obtained from a variety of sources but their identity was reestablished by appropriate serological tests in this laboratory. *Hemadsorption tests.* These were performed as described by Chanock, *et al.* (3). *Hemagglutination tests.* Those conducted with the influenza viruses were carried out with formalinized human erythro-

cytes as previously reported from this laboratory(4). Hemagglutination with the hemadsorption viruses was measured as described by Chanock(3).

Chloroform treatment. It was found that a mixture of 0.05 ml of chloroform and 1 ml of either allantoic or tissue culture fluid containing virus represented a suitable proportion. Although a number of mixing times were tested, it was soon learned that 10 minutes of shaking the chloroform-virus containing fluid was sufficient to kill the susceptible but not the resistant agents. It seemed to be immaterial whether shaking was performed by hand at room temperature or in a mechanical mixer at 4°C. Immediately after shaking, the fluid usually was centrifuged at 400 rpm for 5 minutes. The chloroform then appeared on the bottom of the tube, above this was an opaque, interphase layer, covered by the clear, suspending medium. The latter was removed and used for either egg or tissue inoculation or for hemagglutination. Separation, also, can be accomplished by permitting the chloroform to sediment in tubes standing in a rack. This may be particularly advantageous when there are many preparations to handle.

Results. All of the influenza strains tested (PR-8, FM-1, Asian and B(GL)) proved to be non-infectious after undiluted, infected allantoic fluid was shaken with chloroform and the supernate inoculated into the allantoic cavities of 10-day embryonated eggs. This was also true of a PR-8 strain which had been adapted to grow well in embryo bovine kidney cells(5) (Table I). However, ability of these fluids to agglutinate red cells remained relatively undisturbed, even if exposure to chloroform exceeded 10 minutes (Table II). Such treated virus remained suitable for use in the hemagglutination-inhibition test.

The hemadsorption viruses also appeared to be inactivated by exposure to chloroform.

* Supported by grants from Nat. Inst. of Health, Bethesda, Md.

TABLE I. Effect of Treatment with Chloroform upon Viability of Several Viruses.

Virus titer*				
Virus	Cell substrate	CHCl ₃		Chloroform susceptible
		Treated	Untreated	
Influenza A (PR-8)	Embryo bovine kidney	0	5.0	Sensitive
Parainfluenza† 1	Monkey kidney	0	1.8	"
2	<i>Idem</i>	0	1.2	"
3	"	0	2.1	"
Polio I (Mahoney)	"	7.7	7.7	Resistant
Coxsackie A-9	"	7.9	8.2	"
" B-1	"	6.2	6.7	"
ECHO 4	"	4.7	5.2	"
" 6	"	7.2	7.2	"
" 9	"	6.2	6.7	"
Adenovirus 3	HeLa	5.2	5.2	"
" 4	"	4.9	4.9	"
" 7	"	5.2	5.5	"
Coe	"	6.9	6.9	"
Chavis	"	0	5.7	Sensitive
151 J	Monkey kidney	6.6	6.6	Resistant
M. W.	Hep-2	5.7	5.7	"

* Expressed (except as noted) as log TCID₅₀/ml.

† Parainfluenza virus titers expressed as log HAU/ml.

Another, as yet unidentified, agent which has been obtained from human throats and labelled "Chavis virus" in this laboratory was readily inactivated by chloroform. This agent is also susceptible to ether. On the other hand, poliovirus 1 (Mahoney), Coxsackie A-9 and B-1, echo 4, 6 and 9, adenovirus 3, 4 and 7 and Coe virus all survived exposure to chloroform as they have been reported to do to ether. Viruses 151J and MW recently isolated in this laboratory are resistant to both ether and chloroform. One of these is suspected of being an adenovirus and the other, a member of the echo group.

Discussion. The advantages of being able

TABLE II. Effect upon Hemagglutination Titer of Shaking Influenza Virus (PR-8) for Various Periods with and without Chloroform.

Reciprocal virus dilution	10 min.		30 min.		60 min.	
	CHCl ₃	None	CHCl ₃	None	CHCl ₃	None
10	+	+	+	+	+	+
100	+	+	+	+	+	+
200	+	+	+	+	+	+
400	+	+	+	+	+	+
800	+	+	+	+	+	+
1600	0	+	+	+	0	+
3200	0	+	0	0	0	+

to categorize a virus by a series of relatively simple physical or chemical measures are apparent and have appealed to other investigators. The apparent efficacy of the method described here is particularly attractive. Whether ether susceptible arbor viruses also would be sensitive to chloroform can not be stated on the basis of this study. They should behave in similar fashion unless treatment of infected mouse brain imposes additional requirements as regards exposure time or concentration of chloroform. Whether the immunogenicity of chloroform-sensitive viruses is altered by such treatment also remains to be determined. Davenport, *et al.* (6) recently reported that influenza virus exposed to ether retained its abilities to agglutinate red cells and also was capable of inducing antibodies to inhibit this reaction. The authors suggest that a vaccine made from virus treated in this way might have some advantages over those currently in use. The relative effectiveness of chloroform treated virus in this regard is to be investigated.

Summary. A number of myxoviruses were found to be susceptible to chloroform on short exposure in the same way that they are sensitive to the action of ether. On the other

hand, the adeno, Coxsackie, echo, polio and Coe viruses tested, were resistant to chloroform.

1. Andrewes, C. H., Horstmann, D. M., *J. Gen. Micro.*, 1949, v3, 290.
2. Sunaga, H., Taylor, R. M., Henderson, J. R., *Am. J. Trop. Med. and Hyg.*, 1960, v9, 419.
3. Chanock, R. M., Parrott, R. H., Cook, K., Andrews, B. E., Bell, J. A., Reichelderfer, T., Kapi-

kian, A. Z., Mastrota, F. M. Huebner, R. J., *New England J. Med.*, 1958, v258, 207.

4. Rodrigues-da-Silva, G., Feldman, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1959, v101, 241.
5. Wang, S. S., Feldman, H. A., to be published.
6. Davenport, F. M., Rott, R., Schäfer, W., *J. Exp. Med.*, 1960, v112, 765.

Received February 9, 1961. P.S.E.B.M., 1961, v106.

Effect of Testosterone Propionate on Tissue Protein Synthesis in the Castrated Male Rat.* (26460)

MARCELO E. NIMNI AND LUCIEN A. BAVETTA

Department of Biochemistry and Nutrition, School of Dentistry, University of Southern California, Los Angeles

The fact that nitrogen retention occurs after administration of androgenic steroids to gonadectomized animals is well established (1). This nitrogen retention has been equated with the overall synthesis of total body protein, hence the employment of "anabolic" steroids in attempts to increase overall retention of nitrogen. However, this hormonal induced nitrogen retention has been shown to occur in the castrated rat even during consumption of a protein deficient diet (2) and a positive nitrogen balance could be maintained in spite of a loss of body weight. Furthermore rate of body weight loss was not influenced by the nitrogen retained.

No definite information is available regarding the location of the nitrogen stored by the body during androgenic treatment. The

amount of nitrogen retained is greater than can be accounted for by growth of the sex organs. It would be of interest to localize the sites of protein synthesis which are stimulated by testosterone.

For this purpose we decided to utilize the rate of labeling of tissue proteins of castrated male rats receiving a tracer dose of 1-C¹⁴-glycine as a measure of the anabolic activity of the tissues under consideration.

Materials and methods. Male Wistar rats were bilaterally castrated under light ether anesthesia when they were 2 months old. Fifteen days after gonadectomy they were implanted subcutaneously with sterile polyvinyl sponges (Ivalon), to study the effects of the hormone on rate of synthesis of a newly formed protein such as collagen.

TABLE I. Effect of Testosterone Propionate on Uptake of C¹⁴-Glycine into Protein and Non-Protein Fractions of Tissues in Gonadectomized Male Rats.*

Treatment	Serum	Sem. ves.	Kidney	Heart	Liver	Diaphr.	Perin. musc.	Brain
T.P.	60 ± 8† (1.9)‡	57 ± 11 (8.9)	40 ± 9 (6.3)	15 ± 2 (2.1)	29 ± 4 (3.5)	9 ± 2 (2.9)	15 ± 4 (13.2)	4 ± 5 (1.0)
Control	72 ± 5 (2.3)	16 ± 5 (5.8)	39 ± 10 (7.3)	23 ± 1 (2.7)	39 ± 4 (4.6)	11 ± 3 (4.0)	5 ± 1 (5.5)	3 ± 5 (.6)

* Sacrificed 5 hr after inj. of C¹⁴-glycine.

† Tissue protein specific activity counts/min./mg protein, and stand. error.

‡ Non-protein fraction radioactivity counts/min./mg tissue protein.

* This investigation was supported by U.S.P.H.S. grants.

TABLE II. Effect of Testosterone Propionate on Specific Activity of Protein and Non-Protein Fractions of Tissues and Polyvinyl Sponges of Castrated Male Rats at Different Time Intervals after Injection of C^{14} -Glycine.

Time after inj., hr	Treatment	Serum	Sem. ves.	Kidney	Heart	Liver	Diaphr.	Perin. muse.	Gluteus medius	Brain	Skin	Subcut. sponge
10	T.P. Controls	55* (1.8)† 55 (2.4)	80 (6.9) 19 (3.3)	41 (4.5) 45 (5.0)	21 (2.5) 17 (2.1)	43 (3.5) 43 (6.1)	14 (2.0) 16 (2.2)	21 (5.8) 9 (2.2)	9 (4.2) 9 (4.1)	3 (6) 3 (7)	49 40	53 46
20	T.P. Controls	39 (.6) 41 (.7)	52 (4.3) 11 (2.4)	35 (2.4) 32 (2.2)	14 (3.7) 15 (1.8)	27 (2.4) 24 (1.7)	11 (1.3) 14 (1.4)	22 (4.5) 7 (1.2)	10 (2.5) 9 (2.4)	3 (5) 2 (5)	47 40	51 48
40	T.P. Controls	32 (.5) 31 (.5)	72 (3.8) 21 (2.6)	37 (1.5) 30 (1.6)	23 (1.7) 16 (1.5)	24 (1.2) 26 (.9)	15 (1.7) 23 (1.4)	34 (3.1) 9 (1.1)	11 (2.6) 11 (1.6)	2 (5) 2 (4)	50 43	51 39
60	T.P. Controls	25 (.3) 25 (.5)	42 (1.3) 19 (.1)	27 (1.3) 23 (1.2)	21 (1.5) 19 (1.2)	28 (1.1) 27 (1.2)	13 (1.2) 11 (1.0)	25 (3.4) 12 (.8)	13 (1.6) 11 (1.1)	2 (5) 1 (3)	48 35	45 42

* Specific activity of tissue protein = counts/min./mg protein.

† Non-protein fraction = counts/min./mg tissue total proteins.

Two weeks later they were injected intramuscularly for 3 days with 2.5 mg/day of Testosterone Propionate in 0.25 ml of sesame oil. Simultaneously with the last dose of hormone (or sesame oil in the controls), all animals received intramuscularly 5 μ C of 1- C^{14} -glycine (4.5 mC/mM) in normal saline. They were then sacrificed by overexposure to ether after removing blood samples by cardiac puncture. The organs under investigation were removed and aliquots were homogenized at high speed in a Potter-Elvehjem glass homogenizer using 4% TCA as homogenizing fluid. After centrifugation the non-protein fraction was measured for radioactivity. The protein component was washed following the procedure of Siekevitz(3), and finally plated on aluminum planchets and counted with a gas-flow ultra thin window Geiger-Muller tube. Corrections were made for self-absorption. Skin was previously shaved, then dehydrated and defatted by shaking during two 10-hour periods with acetone and during 24 hours with ether. It was then dried in an oven to constant weight. Subcutaneously implanted sponges were carefully dissected free of surrounding tissue. Both skin and sponges were gelatinized by autoclaving with water at 15 p.s.i. for 8 hours. Collagenous protein was plated and its activity measured as described above.

Results and discussion. Table I shows the distribution of radioactivity 5 hours after injection of the tracer amino acid in the protein and non-protein fractions of different tissues. The highest specific activity is found in the circulating serum proteins of the control animals. The control rats show also a larger uptake of radioactivity by the liver protein fraction. This observation would seem to correlate with *in vitro* experiments where a slight inhibition of protein synthesis occurred in livers of testosterone treated mice(4) and rats(5) and also with the loss of liver mass which occurs in rats receiving this hormone(6).

The organs most significantly stimulated in their protein synthesis were the sex linked ones, namely seminal vesicles and perineal complex muscles.

Table II summarizes the effect of testos-

terone administration on distribution of radioactivity after different time intervals. Again the principal sites of hormonal stimulation were at the level of the sex linked organs. However, some stimulation of protein synthesis of borderline significance appeared to take place in the skin of the hormone treated animals. The high protein synthesizing activity which takes place in the perineal muscle is of interest. This muscle complex includes the levator ani muscle which is widely utilized as a site for measuring myotrophic activity (namely anabolic activity). The behavior of this muscle differs from that of the other muscles under study (diaphragm and gluteus medius) and points to the undesirability of arbitrary selection of an organ for measuring anabolic properties of compounds. It had previously been shown that hypertrophy of the levator ani muscle occurred as a consequence of hormone administration even during the feeding of protein free diets(7).

The results obtained with skin and implanted sponges indicating an increased synthesis of collagen as a result of hormone administration are preliminary. Further experiments will explore the extent and significance of this increased labeling. This observation could be a consequence of an increased turnover of pre-existing collagen molecules induced by testosterone treatment. Boucek *et al.*(8) observed that the rate of labeling of newly formed collagen after injection of radioactive lysine was greater in the male than in the female, although the net synthesis was approximately equal for both sexes.

The value recorded for the radioactivity

present in the non-protein fractions seems to be increased in all the tissues which show a stimulated protein synthesis. Supporting the conclusions of Frieden *et al.*(4), studying *in vitro* uptake of glycine by mouse kidney slices, where it was postulated that testosterone owes at least part of its activity to its efficiency in facilitating intracellular accumulation of amino acids as a preliminary step to protein synthesis.

Summary. Injection of testosterone propionate to castrated male rats stimulated protein synthesis at the level of the sex linked organs, skin, and in subcutaneously implanted polyvinyl sponge. Other tissues studied showed no effect of testosterone on rate of protein labeling after receiving 1-C^{14} -glycine. The unique behavior of the perineal muscle in contrast to other skeletal muscles points to its unsuitability for myotrophic assay of anabolic hormones. The tissues where anabolic activity was stimulated also showed a concomitant increase of radioactivity in their non-protein fractions.

1. Kochakian, C. D., *Vitamines and Hormones*, 1946, v4, 255.
2. Nimni, M. E., Geiger, E., *Endocrinology*, 1957, v61, 753.
3. Siekevitz, P., *J. Biol. Chem.*, 1952, v195, 549.
4. Frieden, E. H., Laby, N. R., Bates, F., Layman, N. W., *Endocrinology*, 1957, v60, 290.
5. Barnelli-Zazzera, A., Bassi, M., Comolli, R., Lucchelli, P., *Nature*, 1958, v182, 663.
6. Selye, H., *Can. Med. Assn. J.*, 1940, v42, 113.
7. Nimni, M. E., Geiger, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1957, v94, 606.
8. Boucek, R. J., Noble, N. L., Woessner, F., Jr., *Ann. N. Y. Acad. Sci.*, 1959, v72, 1016.

Received February 15, 1961. P.S.E.B.M., 1961, v106.

Effect of Dietary Protein and Ascorbic Acid Levels on Biosynthesis of Collagen.* (26461)

L. A. BAVETTA, PAT O'DAY AND ISAAC BEKHOR

Department of Biochemistry and Nutrition, School of Dentistry, University of Southern California, Los Angeles

An important relationship has long been implicated between ascorbic acid and the metabolism of collagen and connective tissue. Hojer(1) reported that in scurvy, collagen formation is diminished and there is a deficiency of collagen in the connective tissue. Abt *et al.*(2) as well as Schauble *et al.*(3) reported that, in guinea pigs, a direct relationship exists between the amount of ascorbic acid in the diet and its concentration in the scar tissue. Gould(4) has demonstrated a direct effect of ascorbic acid on rate of biosynthesis of collagen in the guinea pig. In addition, the literature is replete with experimental studies relating the importance of Vit. C to normal wound healing. Although a need for ascorbic acid in collagen formation is quite evident, the exact mechanism is not entirely clear.

It is well known that rats synthesize ascorbic acid under normal conditions to meet their metabolic needs. However, it is possible that under conditions of stress, such as a low protein diet, synthesis of ascorbic acid or its utilization may be impaired so that rate of collagen biosynthesis is affected.

This experiment was designed to determine the rate of collagen biosynthesis in rats under the influence of different dietary levels of protein with and without extra ascorbic acid supplementation. The biosynthesis of collagen was determined utilizing the sponge technic as employed by Boucek(5). It was assayed at the end of 7, 14, and 21 days after implantation. Collagen was quantitatively estimated by measuring the hydroxyproline content after hydrolysis employing the method of Newman and Logan(6).

Methods. The diets employed contained casein at the 8% and 22% level. The basal ration had the following composition: casein† 8.0; sucrose, 70.5; cottonseed oil, 10.0; Wes-

son salt mix, 5.0; choline chloride, 0.2; and l-cystine, 0.3. To each kg were added the following crystalline vitamins: thiamine hydrochloride, 40 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 100 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; Vit. B₁₂, 150 µg; 2-methylnaphthoquinone, 5 mg; choline chloride, 2 g; Vit. A, 5000 U.S.P. units; Vit. D₂, 500 U.S.P. units; and alpha tocopherol acetate, 100 mg. One of the 8% casein diets and one of the 22% casein diets had 1 g of ascorbic acid added to each kilogram of diet in addition to the amount of ascorbic acid already present in the vitamin mixture employed (0.2 g ascorbic acid per kg of diet). All diets were prepared fresh every 3 days and stored in a refrigerator at 4°C.

One hundred and twenty young male rats of the Holtzman strain, weighing 90 to 110 g were used. The animals were divided into 4 groups of equal weight distribution and each group was placed on one of the above diets. The animals were housed in individual wire bottomed cages and were allowed to eat and drink *ad lib.* during the experiment. All animals were weighed twice weekly.

After one week on their respective diets the rats were implanted with the polyvinyl sponges. One sponge weighing from 100-140 mg was implanted aseptically along the dorsum of each rat. One-third of each group was sacrificed after 7, 14 and 21 days respectively after sponge implantation. The sponge tissue was removed from each animal along with samples of dorsal and ventral skin. Each animal had been shaved with electric clippers to remove hair from the skin areas where the skin samples were taken. All these samples were immediately frozen subse-

* This investigation was supported by U.S.P.H.S. grants.

† Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, Ohio.

TABLE I. Influence of Excess Ascorbic Acid on Biosynthesis Sponge Collagen in the Rat.

	8% casein groups						22% casein groups					
	Wk 1		Wk 2		Wk 3		Wk 1		Wk 2		Wk 3	
	With Vit. C	Without Vit. C	With Vit. C	Without Vit. C	With Vit. C	Without Vit. C	With Vit. C	Without Vit. C	With Vit. C	Without Vit. C	With Vit. C	Without Vit. C
No. of animals	10	8	9	9	7	8	10	10	9	9	9	9
Mean sponge collagen value (mg collagen/g sponge)	37.2	24.0	66.4	61.1	99.0	103.0	66.4	45.7	112.4	85.5	142.3	136.4
Standard deviation	14.6	7.8	12.6	12.6	24	21	19.3	10.1	21.2	13.2	22.4	25.4
Standard error	4.6	2.8	4.2	4.2	9.1	7.5	6.1	3.2	6.7	4.4	7.48	8.46
Difference between Means	13.2		5.3		4.0		20.7		26.9		5.9	
SE _p	5.35		5.9		11.8		6.87		8.0		11.2	
T value	2.5		.9		.34		3.02		3.35		.53	
P "	.02		>.1	<.5	>.5		<.01	>.001	<.01	>.001	>.5	

quent to collagen analysis.

Results. The results of the biosynthesis of collagen in the sponge implants are given in Table I along with the statistical data. After one week the 8% casein group with extra Vit. C added to its diet had a mean value of 37.2 mg collagen per g implanted sponge whereas the 8% casein group without the additional Vit. C had 24.0 mg of collagen per g implanted sponge. The P value between the groups was 0.02, thus there is a significant difference between the collagen values indicating the added Vit. C stimulated collagen biosynthesis during this period. After 2 weeks the 8% casein group with added Vit. C had a mean sponge collagen content of 66.4 mg of collagen per g sponge and the 8% casein group without added Vit. C supplementation had an average content of 61.1 mg per 1 g sponge. There is probably no significant difference between the two groups at the end of the second week or at the end of 3 weeks.

One week after sponge implantation, the 22% casein group with added Vit. C supplementation had a mean sponge collagen content of 66.4 mg collagen/g sponge, while the corresponding group without added Vit. C had a value of 45.7 mg collagen/g sponge. This represents a highly significant difference between the 2 groups. After 2 weeks the Vit. C supplemented group had an average sponge collagen content of 112.4 mg collagen/g sponge whereas the unsupplemented 22% group had a mean value of 85.5 mg collagen/g sponge, a highly significant difference. There is no significant difference between the two groups at 3 weeks. Thus there is a significant increase in biosynthesis of sponge collagen mediated by Vit. C supplementation at the first week with a 8% casein diet and at both the first and second week on the 22% casein diet. The content of collagen in skin of the animals from the various experimental groups is given in Table II. No essential difference between the groups was found. No significant differences were observed in weight increment of the various groups which could be ascribed to supplementation with extra amounts of Vit. C.

TABLE II. Skin Collagen Expressed as Percent Collagen Dry Fat Free Sample.

Casein	Wk 1		Wk 2		Wk 3	
	With Vit. C	Without Vit. C	With Vit. C	Without Vit. C	With Vit. C	Without Vit. C
8%	33.5	35.0	42.0	37.3	42.4	43.2
22%	34.0	32.9	37.2	34.6	37.7	39.4

Discussion. The results of these experiments indicate a direct action of ascorbic acid on the initial biosynthesis of collagen in implanted polyvinyl sponges in the rat. This effect was much more marked under conditions of higher protein intake. It is quite probable that the mechanism for rapid collagen formation requires ascorbic acid as a component or that the vitamin may serve to expedite earlier release of some inhibitory mechanism resulting in rapid biosynthesis of collagen. The negative results obtained on skin collagen may be explained on the basis of the extremely slow turnover of body collagen as compared to such rapid synthesis as that encountered in the experimental system employed.

Summary. Our results indicate that on a diet containing 22% casein, extra amounts of dietary ascorbic acid caused a substantial increment in collagen synthesis which was significant during the first 2 weeks after

sponge implantation. It was also increased by Vit. C supplementation of diets containing 8% casein but the results were less marked than with the higher protein level. The results also indicate that addition of extra amounts of Vit. C to these diets improved their biological value from the point of view of biosynthesis of collagen but not for growth.

1. Hojer, J. A., *Acta Paediatr.*, 1923-1924, v3, suppl. 3, 1.
2. Abt, F. A., Schuching, von S., Roe, H. J., *J. Nutrition*, 1960, v70, 427-437.
3. Schauble, J., Chen, R., Postlethwait, R. W., Dillon, M. L., *Surgery, Gynecology, and Obstetrics*, 1960, v110, 314-318.
4. Gould, S. B., *J. Biol. Chem.*, 1958, v232, 637.
5. Boucek, R. J., Nobel, N. L., *A.M.A. Arch. Path.*, 1955, v59, 553.
6. Neuman, R. E., Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.

Received February 15, 1961. P.S.E.B.M., 1961, v106.

Countercurrent Exchange in Vessels of Renal Medulla. (26462)

NEILS A. LASSEN* AND JAMES B. LONGLEY

(Introduced by G. L. Laqueur)

Laboratory of Pathology and Histochemistry, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

After the proposal of the countercurrent multiplier hypothesis of renal medullary function, Wirz(1) was able to show by micro-puncture experiments that the papillary blood supply was subject to renal countercurrent osmotic concentration and dilution. Berliner *et al.*(2) in restating and modifying the countercurrent hypothesis considered the medullary circulation to be simply a passive countercurrent exchange system working in

parallel with an active tubular countercurrent system. Gottschalk and Mylle(3) have concurred in this view, and presented data confirming Wirz's original observations.

Where a gradient for any diffusible material exists between its 2 ends, a countercurrent exchanger acts as a barrier to the net transport of that material along its long axis. The more diffusible the material, the more effective the barrier. In a passive system concentrations of sodium, chloride, and urea, the principal elements of the papillary hy-

* Present address: Bispebjerg Hospital, Copenhagen, Denmark.

perosmolarity of mammalian kidney, would tend to be conserved more effectively than concentrations of large molecules such as the plasma proteins, but less effectively than gases or other lipid-soluble blood components. Although such an arrangement poses difficulties in terms of gaseous exchange between the deep medulla and the general circulation, the results of the following experiments on incorporation into the papilla of the rat of materials of varying diffusibility are consistent with this simple hypothesis.

Methods. Male Sprague-Dawley rats weighing 200-250 g were used. The animals were kept on normal laboratory diet and had spontaneously hypertonic urine. Incorporation into the renal papilla of radioactive tracer substances of widely different diffusibilities was studied. In some animals only one tracer was employed, in other series 2 tracers were employed simultaneously.

Incorporation of a highly diffusible substance into renal papilla. Radioactive Kr^{85} was used in a concentration of about $50 \mu\text{C}/\text{ml}$ air. The unanesthetized rats were confined in a small glass container with carbon dioxide-absorbing pellets. By injecting oxygen, negative pressure and asphyxia were prevented. At varying time intervals (1, 3, 6, 10, 60 and 90 minutes) the animals were dropped into liquid nitrogen. The frozen animals were sliced transversely on a band saw and autographs prepared by placing X-ray film and the still frozen body sections together at dry ice temperatures. After development, relative Kr^{85} concentrations of the various tissues were estimated by densitometry of the films.

In all animals the cortex of the kidney had approximately the same density on the films as the blood in the heart. This was taken to indicate that the renal cortex is saturated rapidly (in less than one minute) with the inert gas. In the animals sacrificed after 90 minutes of Kr^{85} inhalation autographs of the kidney showed an average papillary density of 93% of the cortical density. This was taken to indicate that the solubility of Kr^{85} in the papilla was 0.93 of that in the cortex. On the basis of these two observations, incorporation of Kr^{85} into the papilla was estab-

lished from the ratio of density of the X-ray film over the renal papilla to that over the renal cortex divided by the factor 0.93.

Incorporation of a colloidal substance into the renal papilla. A qualitative estimate of the state of albumin incorporation after 15 seconds perfusion was attempted. Nembutal anesthetized rats were injected intravenously with about $40 \mu\text{C}$ I^{131} -labeled human serum albumin. Kidneys were ligated at the end of the specified interval, removed, and frozen in dry ice. Radioautographs of unfixed frozen sections were made using ordinary X-ray film.

Simultaneous papillary incorporation of a highly diffusible substance and a colloidal substance. The mode of incorporation of 2 substances of widely different diffusion characteristics was studied in rats using the *in situ* perfusion technic described by Sellers *et al.* (4). The perfusate was about 12 cc of heparinized rat blood at about 25°C . Microcurie amounts of Na^{24} and I^{131} -iodinated human serum albumin or of I^{131} -antipyrine and Au^{198} -colloidal gold were added to the perfusate. A Nembutal anesthetized rat was prepared with one loose tie around each renal pedicle, and a loose tie around the aorta above the renal arteries. A needle of the size of the aorta was inserted into the aorta below the renal arteries. Through this needle the perfusate was introduced rapidly under a hydrostatic pressure of about 140 mm Hg. Simultaneously the aorta was tied above the renal arteries, and the inferior vena cava was cut open. In this way an *in situ* renal perfusion was established. After 10 to 15 seconds, when the supply of perfusate was almost exhausted, both kidneys were tied off, cut free, and placed on dry ice. The renal papilla, a sample of tissue from the renal cortex, and a sample of the perfusate were placed in small vials, and assayed for radioactivity using a well-type scintillation counter. After 2 counts of the samples at separate times, use of the decay factors of the isotopes involved permitted calculation of the original ratio of the two isotopes in each sample.

Results. Incorporation of a highly diffusible substance into papilla. A radioautograph obtained from a rat breathing Kr^{85}

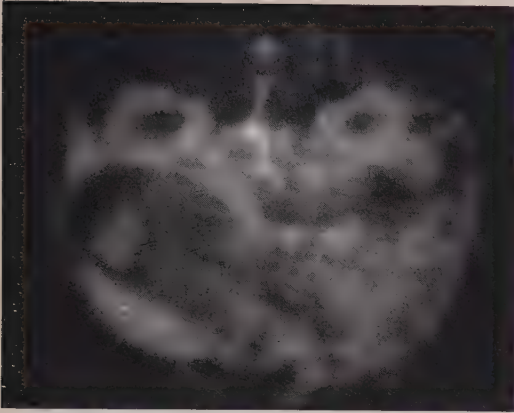


FIG. 1. Autograph, transverse section from rat breathing Kr^{85} for 3 min. Both kidneys (r. and l. at top) show low activity in papillae.

for 3 min is given in Fig. 1. The 2 kidneys are outlined clearly with the cortex standing out as a bright ring. In contrast, the deeper parts of the medulla are quite dark. Thus, relative to the cortex, the papilla is saturated only slowly with Kr^{85} . Results of the densitometer readings from all the animals sacrificed at varying times of exposure are given in Fig. 2.

Incorporation of a colloidal substance into papilla. A radioautograph obtained from a rat kidney ligated 15 seconds after intravenous injection of a small amount of I^{131} -iodinated human serum albumin is shown in Fig. 3. Even at such a short interval incorporation of albumin into papilla is greater than the corresponding incorporation into cortex. Thus incorporation of the large albumin molecule in papilla is, on gross evalu-

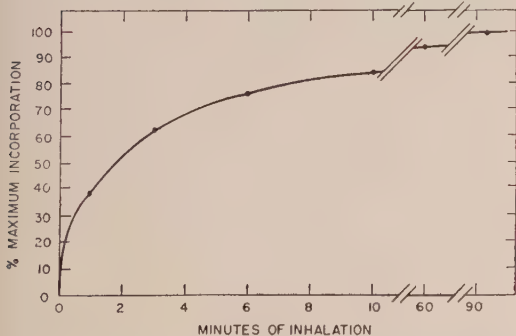


FIG. 2. Rate of incorporation of Kr^{85} into papilla of rat kidney as determined densitometrically from autographs as in Fig. 1.

ation, fast compared to that of highly diffusible Kr^{85} , as presented above. This autograph also shows that the vascular bundles of the inner medulla become distinctly outlined by 15 seconds.

Simultaneous incorporation of a highly diffusible substance and a colloidal substance. Results of aortic infusion experiments using a mixture of Na^{24} and I^{131} -albumin in 8 rats are given in Table I. The autograph of Fig.

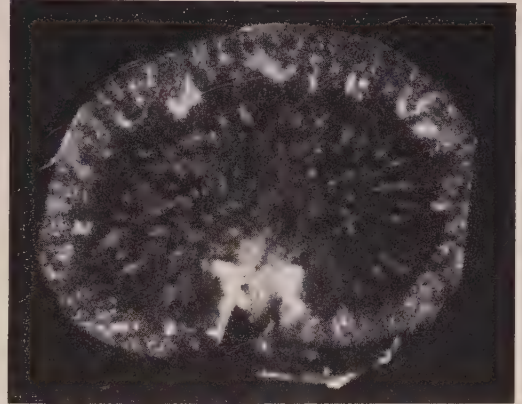


FIG. 3. Autograph, kidney of rat 15 sec. after inj. of I^{131} -human serum albumin. Note activity in papilla. Activity in central portion is in medullary vascular bundles, here cut obliquely. In cortex activity is probably due largely to intraepithelial accumulation of tracer.

3 indicates the distribution of I^{131} -albumin in kidneys of such animals. Autographs of sodium distribution after this interval have been illustrated by Krakusin and Jennings (5.) The quantitative results indicate that under the conditions of these experiments, of the amounts of sodium and albumin available, the cortex incorporates an average of more than 3 times as much sodium as albumin, but in the papilla only about half as much of available sodium is incorporated as of available albumin. Stated in another way, if compared with the simultaneous incorporation of albumin into the two tissues, sodium under the conditions of our experiments is incorporated into the cortex about 6 times as rapidly as it is into the papilla.

Similar calculations on 4 rats perfused with a mixture of I^{131} -antipyrine and Au^{198} -colloidal gold indicate that in comparison to the simultaneous incorporation of colloidal

TABLE I. Relative Incorporation of Na^{24} and I^{131} . Albumin into Renal Cortex and Renal Papilla of Rats (Aortic Perfusion Experiments Lasting 10 to 15 Sec.).

Rat No.	Kidney	Ratio $\text{Na}^{24}/\text{I}^{131}$	
		Cortex/Perfusate	Papilla/Perfusate
1	R	3.03	.24
	L	3.33	.33
2	R	2.94	.69
	L	3.45	.63
3	R	4.00	.75
	L	2.63	.60
4	R	2.63	.42
	L	3.23	.56
5	R	4.35	.42
	L	1.75	.48
6	R	2.77	.65
	L	4.00	.68
7	R	3.87	.48
	L	1.73	.21
8	R	3.74	.45
	L	3.25	.26
Mean values		3.18	.49

gold in the 2 zones, antipyrine enters the cortex 20 times as rapidly as it does the papilla. In 3 of these 4 rats on the same basis the rate of cortical incorporation exceeds papillary incorporation by more than 100 times.

Discussion. Our various experiments are not on a comparable quantitative basis, but the qualitative order of effectiveness of exclusion of the various materials studied seems established. It is reasonable to assume that both serum albumin and colloidal gold have very low rates of diffusibility, essentially zero in comparison to sodium, antipyrine, and krypton. Sodium enters the papilla less readily than albumin, but much more readily than antipyrine does in comparison with colloidal gold. Sodium is therefore excluded with intermediate effectiveness. The observations with krypton cannot be related directly to our other data, but comparing its slow entry into the papilla with the heavy concentration of Na^{22} found by Krakusin and Jennings(5) in the rat papilla within 30 seconds of injection, its exclusion must be regarded as effective compared with that of sodium. Thus the various materials studied are seen to be excluded with an order of effectiveness corresponding to the anticipated

order of permeability of capillaries to each of them. This is what would be expected in a passive countercurrent exchange system. These findings are consistent with those of White *et al.*(6) that water is more effectively excluded from the renal medulla than is sodium.

This conclusion invites comparison once again between the countercurrent system in the renal medulla and the swimbladder in certain fishes. The apparent similarity in both microscopic and submicroscopic structure of the retia mirabilia involved in the 2 cases has been pointed out(7). Attention may also be drawn to the interesting but unexplained tendency of red cells to be absent in the efferent vessels in both cases(7,8). And now, in spite of the seemingly overcomplex fine structures of the retia mirabilia in the rat and toadfish for this function, the objective evidence so far indicates in both cases that only passive countercurrent exchange is involved.

The striking similarity of structure and function in the swimbladder and renal retia mirabilia is even more remarkable when one considers that the countercurrent conservation required in the 2 cases poses significantly different problems. Gases are the most diffusible of the normal molecular population of the blood by a wide margin; their equilibration in a countercurrent system will be achieved almost entirely through exchange of gas molecules, and without any other significant effect on the composition of the blood passing through the rete. On the other hand, the sodium, chloride, and urea constituting the bulk of the osmotic gradient being guarded in the renal papilla are alike in being much less readily exchangeable than gases, and furthermore are also, in other sites, considerably less exchangeable than water (9).

Important consequences follow from this. Whereas conservation of the oxygen gradient across the swimbladder rete necessitates only exchange of oxygen, the effective countercurrent conservation of sodium, chloride, and urea can only be achieved at the expense of severely embarrassed transport of gases into and out of the area of containment, and nec-

essarily involves significant movements of water as well. That this problem of gaseous exchange is a real one in the kidney is shown by our krypton autographs. The low oxygen tension observed in papillary tissue and in urine(10) is probably directly related to it. The high rate of anaerobic glycolysis found for papillary tissue(11) may well represent an adaptive response to an unavoidable and permanent oxygen lack. Speculation on this problem may lead one to ask if any part of the oxygen needs of the papilla might be met by oxygen freed *in situ*, perhaps by a mechanism such as that evidently embodied in the swimbladder gas gland; or, alternatively, whether some modification of the rete vessels in the kidneys does not occur to reduce their permeability to gases and thereby increase the amount of any gas that can be carried the length of the rete. The increase in urinary oxygen tension during water diuresis(12) appears to conflict with both these possibilities, but does not exclude them conclusively.

Given normal characteristics of capillary permeability, equilibration of opposing streams of blood unbalanced with respect to sodium, chloride, and urea will involve not only movements of solutes, but also large bulk movements of water. The direction of this movement in the kidney, as Gottschalk and Mylle(3) have stated, will be such that water will be shunted past the papilla. The expected consequence of this will be that nondiffusible components of the blood will be concentrated to levels above normal in vessels of the papilla. This conclusion has been anticipated for nondiffusible solutes from observations of the tendency of plasma protein to appear in papillary vessels of sectioned material as "colloid"(13), and from tracer determinations of apparent papillary plasma protein "volume" as greater than any likely estimate of the true vascular volume of this tissue(14). Recently Thureau (unpublished results) has shown directly as great as 3-fold concentration of protein over normal plasma levels in samples of plasma obtained by micropuncture from vessels of the hamster papilla. Protein concentration in papillary plasma from this viewpoint occurs only as an un-

avoidable consequence of conserving materials less exchangeable than water by the countercurrent method.

Gottschalk and Mylle(3) reached their conclusion above on theoretical grounds and from observing what they considered elevated red cell concentrations in superficial vessels of the hamster papilla. While the conclusion is evidently correct in the case of plasma proteins, it should be noted that the reported concentration of red cells is in conflict with the conspicuous absence of red cells in the papilla and its efferent vessels in histological preparations(7), the typically pallid appearance of papillary tissue, and with apparent papillary hematocrits of 5% or less obtained by tracer methods(15). A red cell shunt past the papilla even more effective than the shunt for water is demanded to fit these findings. The observation of Gottschalk and Mylle suggests that some of the superficial vessels of the papilla receive their blood from the vessels in the inner stripe (the zone immediately outside the papilla) through which the bulk of the red cells entering the arterioles rectae must be shunted, but should not be taken as evidence of a generally elevated papillary hematocrit.

Summary. 1) The countercurrent action of the renal medullary vasculature, apparently passive, is demonstrated by its differential exclusion of materials from the papilla of the kidney in order of diffusibility. 2) The structural and functional similarity in the rete mirabilia of the kidney and fish swimbladder is stressed. The differences in functional detail in the two cases are proposed to relate to differences in the materials for which gradients are being maintained.

We are indebted to Dr. R. W. Berliner for suggesting the possible significance of a study of rate of incorporation of highly diffusible substances into the renal medulla, and for his continued interest and helpful criticism during the study. The technical assistance of Mrs. H. J. Burtner has been of the greatest value.

1. Wirz, H., *Helv. Physiol. Acta*, 1953, v11, 20.
2. Berliner, R. W., Levinsky, N. G., Davidson, D. G., Eden, M., *Am. J. Med.*, 1958, v24, 730.
3. Gottschalk, C. W., Mylle, M., *Am. J. Physiol.*, 1959, v196, 927.

4. Sellers, A. L., Griggs, N., Marmorston, J., Goodman, H. C., *J. Exp. Med.*, 1954, v100, 1.
5. Krakusin, J. S., Jennings, R. B., *Arch. Path.*, 1955, v59, 471.
6. White, H. L., Tosteson, D. C., Rolf, D., *Fed. Proc.*, 1960, v19, 365.
7. Longley, J. B., Banfield, W. G., Brindley, D., *J. Biophys. Biochem. Cytol.*, 1960, v7, 103.
8. Scholander, P. F., *Biol. Bull.*, 1954, v107, 260.
9. Pappenheimer, J. R., *Physiol. Rev.*, 1953, v33, 387.
10. Rennie, D. W., Reeves, R. B., Pappenheimer, J. R., *Am. J. Physiol.*, 1958, v195, 120.
11. Dickens, F., Weil-Malherbe, H., *Biochem. J.*, 1936, v30, 659.
12. Hong, S. K., Rahn, H., *Fed. Proc.*, 1957, v16, 61.
13. Longley, J. B., Burstone, M. S., *J. Histochem. Cytochem.*, 1958, v6, 89.
14. Lassen, N. A., Longley, J. B., Lilienfeld, L. S., *Science*, 1958, v128, 720.
15. Lilienfeld, L. S., Lassen, N. A., Rose, J. C., *Proc. South. Soc. Clin. Res.*, 1958, 32.

Received September 27, 1960. P.S.E.B.M., 1961, v106.

Fractionation and Immunological Properties of a DNA-rich Preparation from *Brucella abortus*.^{*†} (26463)

OTTO J. PLESCIA, JOSEPH J. NOVAL[‡], NICHOLAS C. PALCZUK AND WERNER BRAUN
Institute of Microbiology, Rutgers University, New Brunswick, N. J.

Previously we described the isolation of DNA-rich preparations from *Brucella abortus* by extraction with 0.5% phenol(1), and the ability of such preparations to produce antisera containing antibodies that precipitated DNase-sensitive antigens(2,3). Subsequent studies(4) revealed that such antisera also contained antibodies to antigens insensitive to DNase treatment, and that only 5% of all immunized rabbits were capable of producing antibodies against DNase-sensitive antigens. This variability may be similar to that encountered in studies of *Lupus erythematosus*, in which antibody-like serum factors reacting with DNA were found irregularly, and to the irregularity with which antibodies to haptens are produced. With this variability in mind, the present report is restricted to an analysis of a lot of DNA-rich material from *B. abortus*, hereafter referred to as preparation #12, which contained DNase-sensitive antigens. Preparation #12,

as well as previously used preparations, consisted largely of DNA, but also contained some RNA, carbohydrate, and about 25% protein that could not be dissociated from the nucleic acid(1). An attempt was therefore made to establish more precisely the chemical basis for the immunological specificity by fractionating the preparation containing the antigens. Progress made in such purification and the properties of the several fractions obtained will be described.

Materials and methods. Immunizing antigens. Preparation #12 ("#12 prep") was obtained from mucoid cells of *Brucella abortus*, strain 19, according to Braun *et al.*(1). It was separated into several fractions by differential ultracentrifugation as described below. *Preparation of antisera.* New Zealand white rabbits (5-6 lb.) were immunized by intravenous injections of a saline solution of either the unfractionated preparation or its fractions. In the course of immunization, each rabbit received a total of 2.5 mg of nucleic acid, as determined by absorption at 260 m μ , or that quantity of a given fraction which was obtainable from 2.5 mg nucleic acid in the unfractionated preparation. Injections were spaced over a period of 4 weeks, with doses of unfractionated material ranging from 0.05 to 0.25 mg of nucleic acid and equivalent amounts of the fractions. The

* Preliminary reports were given at Federation Meetings, Philadelphia, April 1958, and at VII International Congress of Microbiology, in Stockholm, Aug. 1958.

† These studies were supported in part by grants from Damon Runyon Memorial Fund and U. S. Public Health Service.

‡ Charles Pfizer Post-Doctoral Fellow. Present address, N. J. Neuro-Psychiatric Inst., Princeton, N. J.

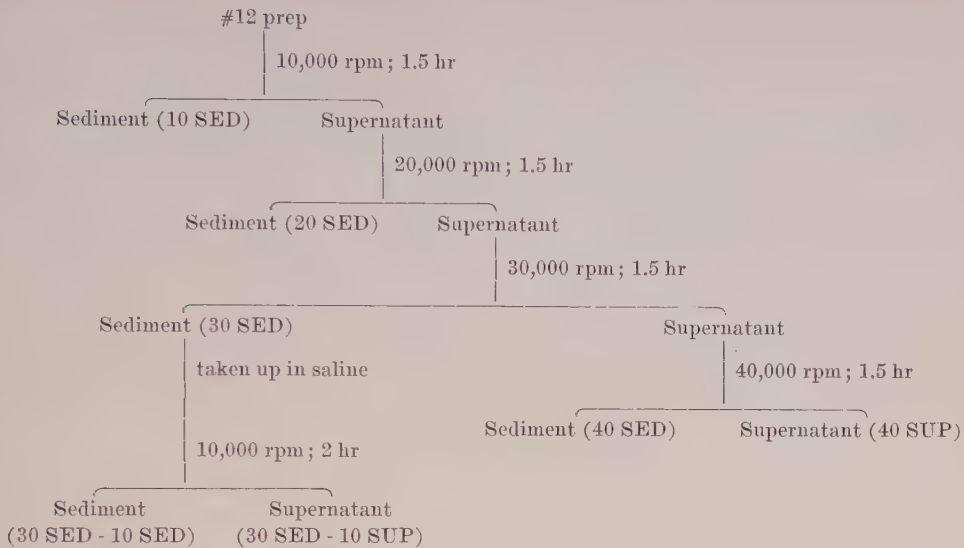


FIG. 1. Fractionation of “#12 prep” by differential centrifugation (Spinco ultracentrifuge, rotor 40, RCF = $6,590 \times G$ at 10,000 rpm).

rabbits were bled by cardiac puncture one week after last injection and the sera were stored at -10°C . *Precipitin analyses.* The technique of Ouchterlony(5) was used for analysis of antigens. Although a series of concentrations of each antigen was tested, only results with optimal concentrations are given. Such solutions contained about 0.5 mg nucleic acid/ml; fractions were used at equivalent concentrations. Antisera were used undiluted or diluted with saline as much as 4-fold.

Results. Fractionation. In preliminary tests “#12 prep” proved heterogeneous electrophoretically, chromatographically, and ultracentrifugally. Differential ultracentrifugation was selected for purification because it gave the best resolution. Fig. 1 is a flow sheet for such fractionation, using a Spinco preparative ultracentrifuge with rotor #40. Each of the fractions, except the final supernatant, was taken up in 0.9% saline to the original volume, and all fractions were analyzed for DNA and protein. DNA was measured in terms of absorption at $260\text{ m}\mu$ as compared to a standard solution of calf thymus DNA(6); however, since independent analyses had established that approximately 5-10% of the nucleic acid represented RNA, the values reported represent maximal

amounts of DNA. Protein concentration was determined on the basis of the biuret reaction as modified by Zamenhof(7). The results (Table I) indicate that protein was not uniformly distributed among the fractions, its concentration being greatest in the fastest sedimenting fraction. The bulk of the protein was found in 10 *Sed* and most of the nucleic acid was in the 40 *Sup* fraction. The ultraviolet spectra of the slower-sedimenting fractions were typical for nucleic acids, whereas that of 10 *Sed* showed no maximum at $260\text{ m}\mu$.

Reacting antigens in fractions. Results of the gel-diffusion analysis are shown in Fig. 2.

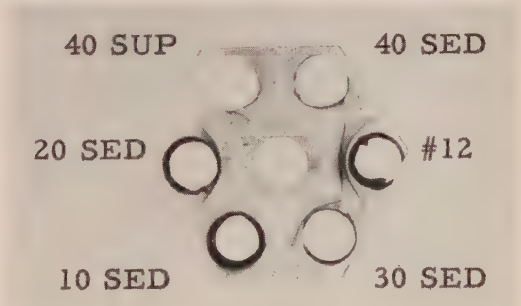


FIG. 2. Analysis of reacting antigens in a DNA-rich preparation from *Brucella abortus*. Center well contains antiserum pooled from 3 immunized rabbits, outer wells contain “#12 prep” and different fractions thereof.

TABLE I. Analysis of Fractions* of “#12 Prep.”

Sample	DNA†		Protein‡		DNA	O.D. (260)	O.D. (260)§
	mg/ml	% of original	mg/ml	% of original	Protein	O.D. (280)	O.D. (230)
#12 (original prep)	1.900	100	.930	100	2.2	1.89	1.56
10 SED	.180	9.5	.530	57.0	.33	1.68	.51
20 "	.122	6.4	.154	16.6	.79	1.83	.90
30 "	.222	11.7	.210	22.6	1.1	1.91	1.91
40 "	.356	18.7	.025	2.7	14.0	1.94	2.14
40 SUP	.922	48.5	.037	4.0	25.0	1.92	2.15
(30 SED - 10 SUP)	.209	11.0	.004	.4	52.0	1.94	2.24
(30 SED - 10 SED)	.060	.6	.200	21.5	.3	1.77	.58

* Cf. flow sheet, Fig. 1.

† Measured by optical density (Englander and Epstein, 1957), “#12 prep” contained 5-10% RNA; therefore these values represent maximal possible values for DNA.

‡ Measured by modified biuret method (Zamenhof, 1957).

§ For native calf thymus DNA, $\frac{\text{O.D. (260)}}{\text{O.D. (230)}} = 2.35$.

The antiserum was placed in the center well; one of the surrounding wells was filled with the immunizing “#12 prep,” and the others with the fractions. The plates were kept at 23°C for 1 week for development. The 10 *Sed* fraction gave no evidence of reaction with the antiserum diluted 1:3 with saline, but precipitated weakly when undiluted antiserum was used in another analysis, indicating a relatively low concentration of antibody directed against antigens in the 10 *Sed* fraction. Both the 40 *Sed* and 40 *Sup* fractions reacted weakly at all concentrations. In the 20 *Sed* fraction some antigens were detectable, whereas the reaction of the 30 *Sed* fraction indicated that it had more of the reactive antigens and in higher concentration. The 30 *Sed* fraction was therefore recentrifuged at 10,000 rpm in an effort to achieve further purification. A sub-fraction, 30 *Sed* - 10 *Sup*, containing less than 2% protein was

obtained. Fig. 3 shows the gel-diffusion analysis of antigens in these sub-fractions. Clearly, all of the reactive antigens were in the DNA-rich fraction 30 *Sed* - 10 *Sup*, and no reactivity was detectable in the protein-rich fraction 30 *Sed* - 10 *Sed*.

The 30 *Sed* fraction subsequently was treated with DNase for 45 min at 26°C, then centrifuged at 10,000, 20,000 and 40,000 rpm for 2½ hr. After such enzymatic treatment, which resulted in a detectable reduction of reacting antigen, it was not possible to sediment remaining antigens even when speeds of 40,000 rpm for 2½ hr were employed.

Immunizing antigens in fractions. Because the reactive antigens were found predominantly in the 30 *Sed* fraction, it was tested for its capacity to elicit the formation of precipitating antibodies in rabbits. A total quantity of 30 *Sed* equivalent to 2.5 mg of 260 mμ-absorbing material in the crude preparation was injected intravenously into rabbits. An equal number of rabbits received the unfractionated preparation. All were bled by cardiac puncture, one week after the last injection, and the sera examined by gel-diffusion. The sera of rabbits injected with the 30 *Sed* fraction, which had most of the reactive antigens, had no detectable precipitating antibody in contrast to the sera of rabbits which had received the crude DNA preparation. This suggests the necessity for

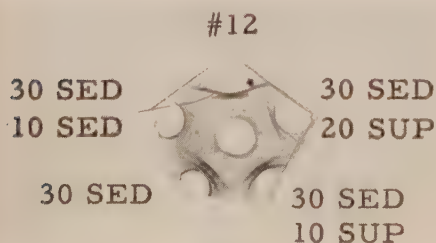


FIG. 3. Analysis of reacting antigens in sub-fractions of 30 *Sed* derived from “#12 prep.” Center well contains the antiserum pool; outer well contains the various fractions.

distinguishing between reactive and immunizing antigens (see discussion).

Rabbits that had received the 30 *Sed* fraction and failed to give precipitins were subsequently injected with a mixture of fractions 10 *Sed* and 30 *Sed*. At the same time, rabbits which had previously yielded precipitating antibodies with the whole "12 prep," and had been allowed to rest until precipitins could no longer be detected in the serum, were given the 10 *Sed* fraction alone. All of the sera now contained precipitins induced apparently by immunizing antigens in the 10 *Sed* fraction but directed against reactive antigens in fractions other than 10 *Sed*, such as 30 *Sed* - 10 *Sup*. Subsequently, previously non-immunized rabbits were injected with the 10 *Sed* fraction alone, and these rabbits also produced precipitating antibodies directed against antigens in fractions other than the immunizing fraction. It seems, therefore, that the protein-rich fraction 10 *Sed* can contribute the immunizing antigens, but that the antigens reacting with immune sera are to be found among the DNA-rich fractions.

Discussion. These fractionation studies have demonstrated that in antigenically active DNA-rich preparations, obtained by 0.5% phenol extraction, protein is not uniformly distributed among fractions obtained by differential ultracentrifugation. This method of fractionation, therefore, has proved useful for an analysis of antigenic properties associated with materials differing markedly in their ratio of DNA to protein.

Reacting antigens were concentrated predominantly in fractions rich in DNA and were not proportional to the relative amounts of protein present. Thus the protein-rich subfraction of 30 *Sed* contained no reacting antigens, whereas the DNA-rich supernatant 30 *Sed* - 10 *Sup* showed undiminished reactivity. Furthermore, reactivity was not abolished by the action of proteolytic enzymes such as trypsin, chymotrypsin and panprotease. However, since the purest available preparations still contain traces of protein, the latter cannot be excluded definitely as a necessary part of the reactive antigens.

The likelihood that at least some of the several antigens present in the 30 *Sed* fraction are in some manner associated with DNA is indicated by the finding that DNase treatment of the 30 *Sed* fraction diminishes the intensity of the antigen-antibody reaction and decreases the molecular weight of the antigens so that they can no longer be sedimented at 40,000 rpm. The possibility that the antigens are complexes containing DNA has been suggested by our observation that treatment of the reacting antigen preparation with periodate, which acts primarily on carbohydrates but not on deoxyribose of DNA, results in a complete abolition of antigenic reactivity.

The protein-rich 10 *Sed* fraction, which was capable of stimulating the formation of precipitating antibodies that reacted with the 30 *Sed* - 10 *Sup* fraction (containing less than 2% protein), did not appear to have reactive antigens. Furthermore, the reactive fraction, 30 *Sed* - 10 *Sup*, was unable to stimulate production of precipitating antibodies. It would appear, therefore, that a distinction must be made between the immunizing and reactive antigens. The failure to elicit antibodies with the relatively pure DNA fraction is consistent with the general experience of others who could not detect precipitating antibodies in the sera of animals injected with pure DNA(8). As yet no experimental data are available to explain the differences noted in the immunogenic capacities of purified DNA *vs.* DNA-protein.

While these studies have failed to yield pure antigens and thus have not enabled us to associate the DNase-sensitive antigens with a well-defined chemical entity, they have indicated at least that substantial concentrations of protein do not play a role in the antigen-antibody reactions here studied. Even though the specificity of antigens associated with DNA may not ultimately depend upon DNA itself, such antigens, nevertheless, would appear to be of considerable interest chemically and, above all, biologically.

Summary. A DNA-rich preparation, obtained from *Brucella abortus* by extraction with 0.5% phenol, was fractionated by differential ultracentrifugation, and its immuni-

logical properties were studied. The antigens that precipitated antibodies from the sera of rabbits injected with the crude DNA preparation were concentrated in a fraction consisting essentially of DNA and only about 2% protein. This fraction did not elicit the formation of precipitating antibodies, whereas a protein-rich fraction with no reactive antigens did. Treatment of the reactive antigens with DNase altered some of their properties. On the basis of present evidence, it is suggested that the DNase-sensitive antigens are complexes containing DNA.

1. Braun, W., Whallon, J., Phillips, J. H., *Nature*, 1957, v180, 1356.

2. Phillips, J. H., Braun, W., Plescia, O. J., *ibid.*, 1958, v181, 573.

3. ———, *J. Am. Chem. Soc.*, 1958, v80, 2710.

4. Braun, W., Plescia, O. J., Palczuk, N. C., *Proc. 5th Intern. Congr. Biol. Standardization*, 1959, 367.

5. Ouchterlony, O., *Ark. Kemi Min. Geol.*, 1948, v26B, 1.

6. Englander, S. W., Epstein, H. T., *Arch. Biochem. and Biophys.*, 1957, v68, 144.

7. Zamenhof, S., in Colowick, S. P., and Kaplan, N. O., eds. *Methods in Enzymology*, Academic Press, N. Y., 1957, vIII, 702.

8. Levine, L., Murakami, W. T., Van Vunakis, H., Grossman, L., *Proc. Nat. Acad. Sci.*, 1960, v46, 1038.

Received November 3, 1960. P.S.E.B.M., 1961, v106.

Effect of ACTH on Sex Ratio of the Albino Rat.* (26464)

ERICH GEIRINGER

Department of Medicine, University of Otago, Dunedin, N. Z.

Mammalian spermatogenesis results in an equal number of male and female producing spermatozoa. The actual sex ratio of most species, however, differs characteristically from the expected theoretical value of unity (1).

The factors which enter habitually into the reproductive process of those species and modify the sex ratio (number of males per 100 females) could be paternal, e.g. excess mortality of one type of spermatozoon after spermatogenesis or diminished aptitude of one type of spermatozoon for fertilization. Such and other differential properties of the 2 types of spermatozoa have been demonstrated and have become the subject of considerable research. On the other hand, the maternal milieu interne could be such as habitually to favor survival of, or fertilization by, one type of spermatozoon. These two possibilities may be largely synonymous.

In addition to these normal and species

specific deviations from unity of the sex ratio further changes in sex ratio occur in several well authenticated situations. The old observation that the human sex ratio rises in war time was again verified during the second world war and statistical analysis suggests that this rise was not due to age of the parents or to the birth order(2). In many species including man, it has also been noted that the first born or the first litter tends to have a significantly higher sex ratio than the species as a whole. In the rat with a species specific sex ratio of 105 the ratio for first litters is 122(3). In some animals (cattle, sheep, pigs, wolf hounds) statistics indicate that the relative number of female births tends to rise during the cold months of the year. King has confirmed this for the rat(3).

These observations suggest that stress may be able to alter sex ratio. The possibility that the adrenal cortex may be involved in mediating these effects is strengthened by 2 observations of King and Donaldson made in 1929, i.e. before the relation between adrenal cortex and stress responses was understood. King recorded the sex ratio of the

* Supported in part by the British M.R.C. My thanks are due to Mr. George F. Spears, Otago Medical School, for statistical evaluation of the results, and to Mrs. Evelyn Ewing and Miss Maureen Kennedy for technical assistance.

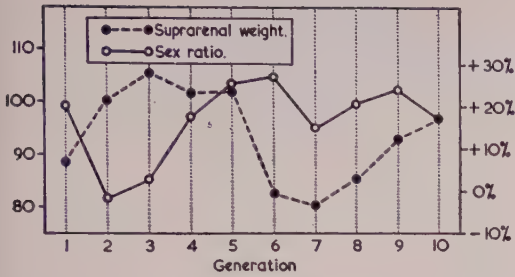


FIG. 1. Relation of adrenal wt to sex ratio in 10 generations of Gray Norway Rats. (Adapted from King and Donaldson, *Am. Anatomical Memoirs* No. 14.) Figures on left give No. of males per 100 females; figures on right, % increase of adrenal wt over standard adrenal wt for albino rats.

gray Norway rat during 10 generations in captivity while Donaldson showed changes in adrenal weight in the same animals over the same period(4). I have superimposed these 2 graphs upon one another to show the striking inverse relation between them (Fig 1). It is seen that rises in adrenal weight correspond to falls in sex ratio.

Design of Experiment. The animals were female albino virgin rats. Female litter mates, kept in the same cage and under the same conditions throughout experiment served as controls. At the end of each experiment the experimental animals and their litters were killed and checked for abnormalities whereas control litters were allowed to grow up for further experiment. Insofar as inherited tendencies are involved, the experiment was deliberately designed with a bias against production of differences between control and experimental groups, since all experimental groups except the first were the offspring of control animals.

Control animals were given .05 ml saline, experimental animals .05 ml (1 unit) long acting ACTH subcutaneously every second

day. On the day after first injection the animals were mated with a normal untreated male of the same strain. At the end of one week both injections and mating were interrupted for one week to minimize any possible effect of ACTH on pregnancies. Pregnant animals were isolated, and injections and mating were resumed with the remainder. This weekly alternation was continued until all animals had become pregnant or until 6 weeks had elapsed. Sex ratio of the litters was recorded as soon as possible after birth. The same sire was used for as many succeeding experiments as possible. Only 6 sires were utilized throughout the investigation.

The investigation was carried out in 2 series. In the first series (in Glasgow) Corticogel (Crookes) was the ACTH used. The second series (in Dunedin) was carried out in the same manner except that Cortrophin ZN (Organon) was used.

Results are given in Table I. They are essentially the same in both series, which may therefore be considered together. ACTH medication had no adverse effects on fertility. The number of animals which failed to be fertilized within 6 weeks was very small and roughly equal in both control and experimental groups. Average litter size compares well with that recorded for large well looked after colonies and is the same in both control and experimental groups. Sex ratio at birth, however, is significantly depressed in the treated animals. This is seen both in the aggregate figures and in proportion of predominantly female litters. The change is a generalized shift and not a clear cut all-or-none phenomenon.

Discussion. It is reasonable to assume that this effect is produced after the male

TABLE I.

	Series 1		Series 2		Total	
	Control	Treated	Control	Treated	Control	Treated
No. of animals born	114	186	293	183	407	369
" " litters	15	25	33	19	48	44
Avg size of litters	7.6	7.3	9	10	8.47	8.38
No. of predominantly female litters	3	13	12	10	15	23
Males : Females	68:46	89:97	159:134	81:102	227:180	170:199

$$\chi^2 = 9.24, \text{ i.d.f., } .001 < P < .01.$$

TABLE II. Distribution of Litter Sizes.

No. in litter	Control	Treated	No. in litter	Control	Treated
3	1	2	9	11	7
4	0	1	10	10	7
5	3	2	11	3	6
6	4	3	12	2	1
7	2	5	13	0	1
8	12	9			
			Total No. of litters		
			48 44		

ejaculate has entered the maternal passages. Is it produced before or after fertilization of the ovum? Does ACTH act by a differential effect on foetal mortality? If ACTH increased male foetal mortality, the experimental litters should be smaller, and if it acts by reducing female foetal mortality they should be larger than control litters. Average litter size is the same in both groups. If ACTH acts simultaneously in both directions the effect should be most noticeable in the smaller litters. Experimental litters with less than 10 animals were found to have a sex ratio of 95 against one of 77 for litters with more than 10 animals. It is therefore unlikely that the ACTH effect was on foetal mortality. Table II showing the very similar distribution of litter size in the 2 groups does not suggest that litter size was in any way connected with the ACTH effect.

Another possibility, namely that the change in overt sex ratio may not correspond to the nuclear sex ratio i. e. that ACTH may have produced females with male nuclear sex cannot be excluded. However, no malformations were observed in any of the experimental animals and there is nothing in the literature to suggest that complete reversal of the genotypic sex can be produced in offspring by moderate stimulation of the mother's adrenal cortex.

The most reasonable hypothesis seems to be that the effect was on primary sex ratio,

i. e. on number of ova fertilized by either type of sperm. The vaginal, uterine or ovarian environment seems to have been modified so as to increase the chances of female producing spermatazoa to fertilize.

Is this effect mediated through the adrenal cortex or is it a non-specific effect of ACTH or of one of its impurities, diluents, or suspending agents? In view of King's observations (Fig. 1) and in view of the use of a different type of ACTH in the 2 series the former seems more likely.

The size of the sex ratio depression obtained more than abolishes the species specific bias towards male conceptions shown by the rat, but the effect of ACTH will not necessarily be in the same direction in other species.

The animals used were young virgin females and to some extent the effect of ACTH resembles therefore that of normal maturation, since the sex ratio of first litters tends to be high in the rat. However, ACTH depresses the sex ratio much more than would be compatible with a mere speeding up of maturation.

Summary. Subcutaneous administration every second day of one unit of long acting ACTH to female albino rats before and during mating significantly depresses the sex ratio at birth of their first litters.

1. Goldschmidt, R., *The Mechanism & Physiology of Sex Determination*, London, 1923, pp. 220, 251.

2. McMahon, B., Pugh, T. F., *Am. J. Human Gen.*, 1954, v6, 284.

3. King, H. D., Stotsenburg, J. M., *Anat. Rec.*, 1950, v9, 403.

4. Donaldson, H. H., *American Anatomical Memoirs* (14) Philadelphia 1929, Life Processes and Size of the Body and Organs of the Gray Norway Rat During Ten Generations in Captivity.

Received December 1, 1960. P.S.E.B.M., 1961, v106.

Cultivation of Duck Hepatitis Virus in Tissue Culture.* (26465)

M. L. KAEBERLE, JOHN W. DRAKE AND L. E. HANSON

(Introduced by C. A. Brandy)

University of Illinois, Department of Veterinary Pathology and Hygiene and Department of Microbiology, Urbana

A viral disease of ducks characterized primarily by hepatitis was first described by Levine and Fabricant(1). The viral agent and course of the disease have been studied by several investigators(2-6). Incentive to study this virus has been provided by the economic importance of the condition to the duck industry and possible relationship with viral hepatitis in other species.

Investigation of the virus of duck hepatitis (DHV) has led to attempts to cultivate the agent in other hosts. The virus multiplies readily in embryonated chicken eggs to which it has been adapted(1). Pollard and Starr (7) attempted to propagate the virus in chicken embryo explants and trypsinized chicken embryo cell cultures. The virus multiplied in explant cultures, but persisted through only 3 passages in trypsinized cell cultures.

The reported success in growing trypsinized liver cells in tissue culture(8,9) suggested a promising host for the virus. This paper reports results of cultivation of duck hepatitis virus in liver tissue culture cells.

Materials and methods. *Virus.* DHV strain R85952(3) which had been passed serially 18 times in embryonated chicken eggs was used for this study. Allanto-amniotic fluid from embryos which died following viral inoculation served as a source of virus. Virus was stored at -20°C .

Viral assay and identification. Viral titer of allantoamniotic fluid and tissue culture material was determined by infectivity for 8-day-old embryonated chicken eggs. Identification of virus was established by neutralization with duck serum known to contain DHV neutralizing antibodies. Gel diffusion precipitation tests(10) utilizing anti-DHV rabbit serum served to identify virus

and provided a semiquantitative measure of viral content.

Tissue culture. Livers of 16-day-old chicken embryos were removed aseptically, minced with scissors, and trypsinized at 39°C with 0.25% trypsin (Difco 1:250) in Earle's saline (ES). Cells were washed, once in cold ES and once in nutrient medium, and 2 ml of a 1.5% suspension of cells in nutrient medium were placed in 16×150 mm rubber stoppered tubes. Nutrient medium consisted of 30% inactivated sheep serum, 10% chicken embryo extract or 15% beef embryo extract, 50 mg/ml dihydrostreptomycin sulfate and 100 units/ml penicillin added to Eagle's medium. Tubes were incubated at 37°C in a stationary rack positioned at a 5° angle.

Inoculation of tissue culture with virus. When cells had formed a confluent sheet (about 4 days) they were inoculated with virus. Nutrient medium was removed and 2 ml of fresh medium and 0.2 ml of viral suspension were added. Tubes were incubated without a change of medium for 6 days, at which time they were frozen at -20°C .

Cytopathologic study. Cells were observed daily for signs of abnormality. Coverslips were removed periodically from tubes and cells were stained by the May-Grünwald-Giemsa technic. Stained cells were examined microscopically for signs of cytopathogenic effects (CPE).

Results. Cell cultures derived from chicken embryo livers were composed of at least 3 cell types: fibroblasts, epithelial-like cells and polygonal cells with small, round nuclei. The latter cells were very similar histologically to hepatic cells and tended to grow in islands surrounded by the other cell types (Fig. 1). Polygonal cells characteristically contained lipoidal inclusions, and numerous mitotic figures could be observed. Fibroblasts overgrew other cell types and, if

* This project supported by NIH grant and Illinois Agri. Exp. Sta.

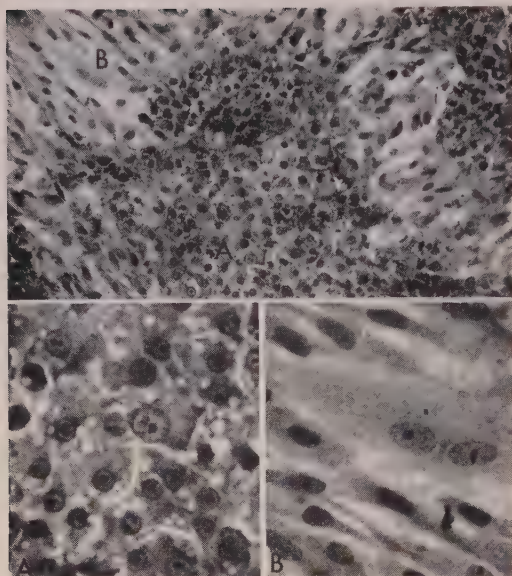


FIG. 1. Uninoculated chicken embryo liver tissue culture. Note islands of "hepatic" cells (A) surrounded by cells with fibroblastic and epithelial morphology (B). May-Grünwald-Giemsa stain. Magnification 100 \times and 300 \times respectively.

their multiplication was not restricted, excessive growth caused peeling of the cell sheet. Embryo extract incorporated in nutrient medium served to restrict fibroblast proliferation.

Assay of each serial passage of virus in tissue culture demonstrated a decrease in viral titer from passages 1 to 6 and a moderate rise in the 7th passage as shown in Table I. Although titer of virus dropped consider-

TABLE I. ID_{50} Titers of Tissue Culture Virus for Embryonated Chicken Eggs.

	T.C. passage						
	1	2	3	4	5	6	7
Titer*	$10^{8.8}$	$10^{2.5}$	$10^{1.6}$	$10^{1.0}$	$10^{1.7}$	$10^{0.9}$	$10^{2.9}$

* Calculated by method of Reed and Muench(11).

ably from that in allanto-amniotic fluid it is quite obvious that virus did multiply in tissue culture medium (See Discussion).

Gel diffusion precipitin test demonstrated presence of virus (Fig. 2). Observable lines of precipitate developed within 12 hours with viral passages 1, 2, 6, and 7. Faint lines of precipitate formed with passages 3, 4 and 5 after reservoirs were refilled several times

with antigen and incubation was continued for 24 to 36 hours. Controlled tests demonstrated the specificity of antigen present in tissue culture material.

Microscopic examination of liver and stained tissue culture cells failed to reveal CPE. No changes in character of cells or their multiplication were seen during passage of the duck hepatitis virus.

Discussion. Limited multiplication of DHV was demonstrated in chicken embryo liver tissue culture cells. Multiplication would have been necessary for virus to be present in terminal passages since dilution and decay of virus at 37°C would have reduced the amount of virus below the threshold of detection. Pollard and Starr(7) have reported a decrease in titer between 10^2 to $10^{5.5}$ fold in several tests when the virus was incubated at 37°C for 21 days. The 42-day incubation period, taken together with a total dilution factor of 10^7 over 7 passages, would have totally removed original input virus. The marked drop in viral titer during the first few passages followed by an increase in the 7th passage suggests that the virus was becoming adapted to tissue culture cells. Earlier development of lines of precipitate with the gel diffusion precipitation test in initial and terminal passages was also probably related to viral concentration thereby indicating multiplication of virus.

Since marked histopathologic changes are seen in infected chicken embryos and ducks (4), failure of virus to induce cytopathologic changes in tissue culture cells was not expected. However, mouse hepatitis virus(12), as well as a number of other viruses(13), can

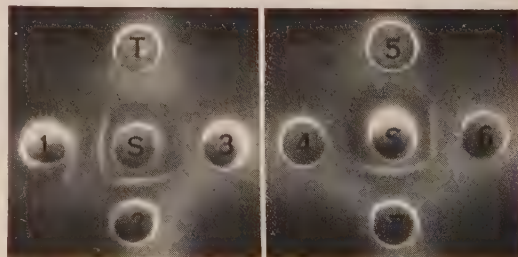


FIG. 2. Gel diffusion precipitation with duck hepatitis virus from various tissue culture passages. Numbers indicate passage level, T represents uninoculated tissue culture, and S stands for rabbit anti-duck hepatitis serum.

multiply in tissue culture without visible CPE. Pollard and Starr(7) also were unable to detect histopathologic changes in chicken embryo explants that supported multiplication of DHV. Apparent lack of CPE may be a reflection of a low number or percentage of infected cells rather than an indication of viral multiplication without CPE. Observed titers could conceivably have been produced by release of a few hundred infective virus particles from as few as 10 cells per ml, a number virtually undetectable among the far larger number of cells in each tube.

Summary. Duck hepatitis virus was passed serially 7 times through monolayer chicken embryo liver cell cultures. Multiplication of virus was demonstrated by assay in embryonated chicken eggs and by gel diffusion precipitin test. Microscopic examination of stained tissue culture cells failed to reveal CPE.

1. Levine, P. P., Fabricant, J., *Cornell Vet.*, 1950, v40, 71.

2. Asplin, F. D., McLauchlan, J. D., *Vet. Rec.*, 1954, v66, 456.

3. Hanson, L. E., Alberts, J. O., *J. Am. Vet. Med. Assn.*, 1956, v128, 37.

4. Hanson, L. E., Ph.D. Thesis, Univ. of Illinois, 1957.

5. Macpherson, L. W., Avery, R. J., *Canad. J. Comp. Med.*, 1957, v21, 26.

6. Asplin, F. D., *Vet. Rec.*, 1958, v70, 393.

7. Pollard, M., Starr, T. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v101, 521.

8. Sharpless, G. R., Defendi, V., Cox, H. R., *ibid.*, 1958, v97, 755.

9. Fontes, A. K., Burmester, B. R., Walter, W. G., Iseler, P. E., *ibid.*, 1958, v97, 854.

10. Murty, D. R., M.S. Thesis, Univ. of Illinois, 1960.

11. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

12. Starr, T. J., Pollard, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v100, 97.

13. Deinhardt, F., Henle, G., *J. Immunol.*, 1957, v79, 60.

Received December 9, 1960. P.S.E.B.M., 1961, v106.

Cultivation *in vitro* of Adult Human Skin and Oral Mucosa on Gelatin Film.* (26466)

JEROME B. SMULOW,[†] ROBERT RUSTIGIAN, AND MAURAY TYE
(Introduced by Louis Weinstein)

*Departments of Microbiology, Oral Pathology and Periodontology, and Dermatology,
Tufts University School of Medicine and Dental Medicine, Boston*

Successful cultivation of human skin *in vitro* has been reported by numerous investigators(1-8), the majority of whom employed the plasma clot technic with small fragments of skin obtained from either living donors, or from cadavers. Plasma clot may present certain disadvantages for immunological, nutritional(9) and viral studies(10). Evans and Earle(9) have used perforated cellophane as a substitute for plasma clot for growth of some tissues. It would appear, however, that

perforated cellophane is not a satisfactory substrate for primary cultivation of adult human skin(4). Attempts in this laboratory to cultivate single specimens of adult human skin from 9 donors with perforated cellophane were unsuccessful. Puck *et al.*(7) have used the method of trypsinization for successful growth of cells from fresh adult skin and other human tissues. Excision of 20 to 50 mg of skin(11), however, is not always feasible when repeated fresh specimens, particularly from the same area, are desired for cultivation. Ehrmann and Gey have reported growth of a number of cell strains and fresh explants of human tissue on transparent collagen gels(12) and Enders(13) has

* These studies were supported by grants from Nat. Inst. Health, Divisions of Allergy and Infect. Dis. and Dental Research, U. S. P. H. S.

[†] Research Fellow in Oral Pathology and Periodontology.

observed growth of cells from human embryonic tissue to occur on absorbable gelatin film which is available commercially.[‡]

This report is concerned with observations on cellular outgrowth from single small fragments of adult human skin on gelatin film as compared to plasma clot technic. Similar studies were carried out with adult human oral mucosa which to our knowledge has not been cultivated *in vitro*. There is some question as to whether the appearance of epithelioid cells from fragments of adult human skin represents cell migration or multiplication (2, 5). No attempts were made to clarify this question and accordingly the term, "growth," as applied to these cells, is used with this reservation in mind.

Materials and methods. One hundred and seventy-nine specimens of human skin[§] were obtained from 72 donors most of whom were personnel or students at Tufts University ranging in age from 19-63 years. Fragments were usually taken from the lateral surface of the upper part of the right arm which was washed with sterile saline and the point of a 25 gauge needle was inserted very superficially into the skin. This was used to gently lift and hold the skin while a #11 Bard-Parker blade was placed just beneath the needle point and the thin layer of skin quickly excised. Stained histologic sections of representative specimens revealed some dermis as well as epidermis (Fig. 1). The specimens weighing from 0.15-0.40 mg and measuring from 1-2 mm were placed in about 4 ml of saline A(7) for one to 2 minutes before setting up for culture. Sixty-nine oral mucosa specimens were taken from the lower labial region of 32 different donors. The area was dried with sterile gauze, the tissue was lifted gently with small blunt forceps, and a fragment excised with a #11 Bard-Parker blade. Mucosal sections were not weighed but were similar in size to the skin fragments.

Plasma clot technic. A drop of chicken plasma (Difco dehydrated) was added to a

#1 coverslip (22 mm²) in a 60 mm Petri dish and a single fragment of skin or oral mucosa was placed in the plasma. No attempt was made to orient either surface of the tissue to the glass. One to 2 drops of 50% chick embryo extract in Hanks' salt solution (W/V), adjusted to pH 7.0 with NaOH, was then added to induce clotting which occurred in from 5 to 20 minutes. Three ml of medium (described below) were placed in the culture dish, which was then incubated at 36-37°C in an atmosphere of approximately 5% CO₂ in air.

Gelatin film technic. Pieces of gelatin film (about 10-20 mm²) were cut from a sheet (100 × 125 mm and 0.075 mm in thickness) and sterilized at 140°C for 4 hours. These were washed 3 times with Hanks' salt solution containing 0.05% NaHCO₃ to neutralize the acidity of the gelatin film. The skin fragment was placed in a sterile 60 mm Petri dish with the keratin surface facing the glass. The dermal portion could be recognized as a domed white surface in contrast to the darker and flatter corneum. The square of prepared gelatin film was placed over the specimen in contact with the dermal surface of the fragment. If this were reversed, no growth or poor growth occurred almost without exception.

Media. The media consisted of Eagle's basal solution supplemented with 20% human serum (EHu₂₀ medium), 10% human serum and 5% calf serum (EC₅ Hu₁₀ medium), and 15% calf serum (EC₁₅ medium). Eagle's solution was modified by addition of inositol(14) and replacement of Earle's salt solution with Hanks containing 0.05% NaHCO₃. Human serum consisted of a pool obtained from 10-12 young adult donors collected under sterile conditions and stored in small aliquots at approximately -25°C. Stored serum, after thawing, was used either immediately or held at 5°C to 10°C and used as needed over a period of about 2-3 weeks. Calf serum pools were obtained from the blood of 30-40 calves slaughtered at a local abattoir and sterilized by filtration through a Seitz filter which was first washed with 800 ml of distilled water and 200 ml of serum pool. It was stored and used in the

[‡] Gelfilm, Upjohn Co., Kalamazoo, Mich.

[§] A few of these skin specimens and several specimens of oral mucosa were cultured by Dr. William Gibson as part of another study to be reported later.

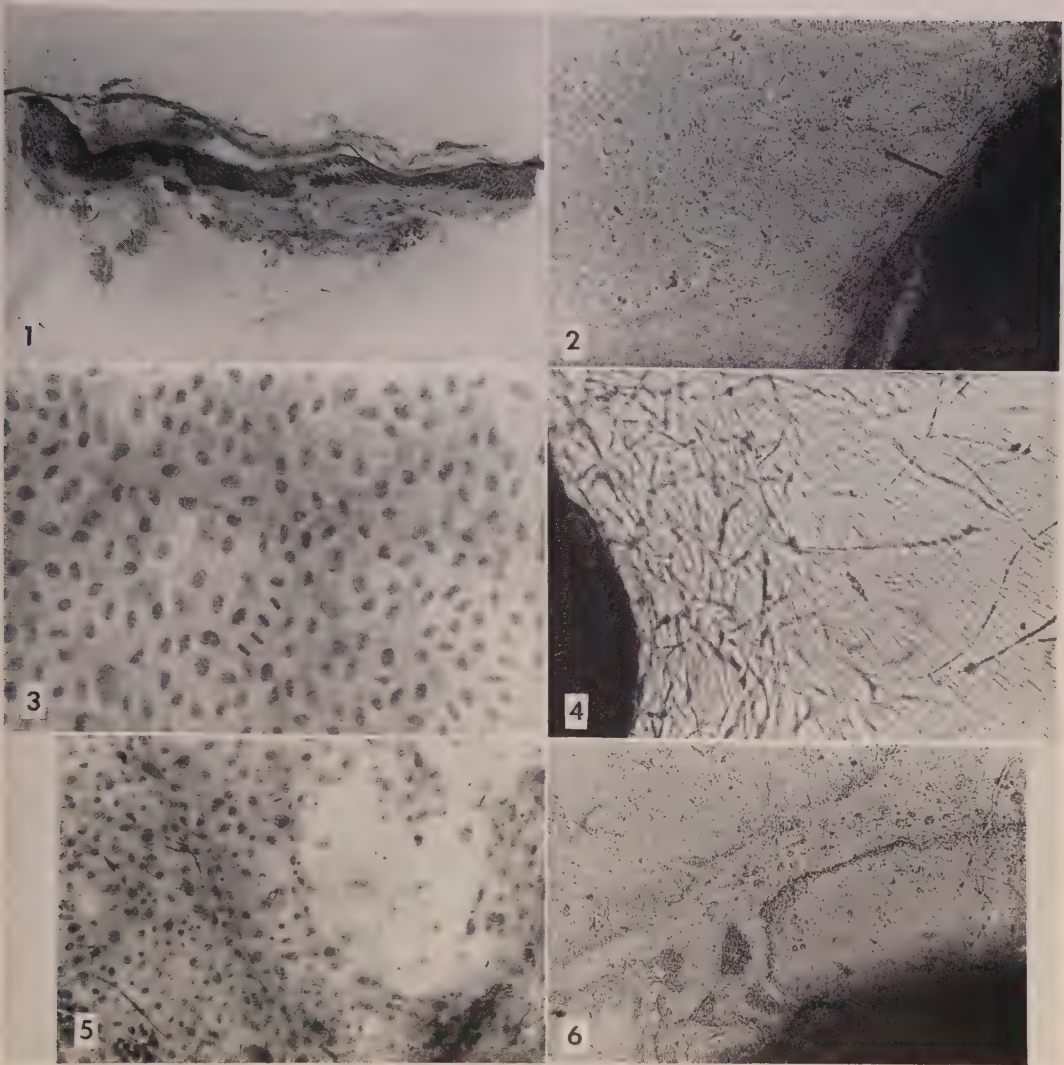


FIG. 1. Histologic section of skin fragment representative of specimens used for culture, showing epidermis and corium. Hematoxylin and Eosin. $\times 50$.

FIG. 2. Skin fragment grown on gelatin film for 21 days showing epithelioid cell outgrowth. Unstained. $\times 50$.

FIG. 3. Same as Fig. 2 after Hematoxylin and Eosin staining. $\times 215$.

FIG. 4. Fibroblast-like cells growing from section of adult human skin cultured in plasma clot for 16 days. Note appearance of hyphae-like forms and typical compressed spindle forms in uniform sheath. Unstained. $\times 50$.

FIG. 5. Outgrowth from adult human skin containing area of large pale cells with poorly stained cytoplasm bordered by characteristic epithelioid cells. Hematoxylin and Eosin. $\times 50$.

FIG. 6. Epithelioid cells from adult oral mucosa. Eighteen-day-old culture. Hematoxylin and Eosin. $\times 50$.

same manner as human serum. Soybean trypsin inhibitor(15,16) was added to all media to a final concentration of 0.05 mg per ml. Penicillin, streptomycin and mycostatin were added in final concentration of 100 units, 100 μ g and 50 units per ml respec-

tively. Initial pH of the media was 7.9 to 8.4. After 7 hours incubation under 5% CO₂ it dropped to 7.2-7.4 and remained more or less constant when tested on 3 successive days. Cultures were examined at 2 to 4 day intervals at which time fluid changes

were made. The majority of the cultures were held for 3-4 weeks in plasma clot and 4-6 weeks under gelatin film.

Staining of cultures. Cultures were washed for 15 minutes in 3 changes of saline A. Sections were then fixed in 10% formalin for 2 hours and stained with hematoxylin and eosin. Although the plasma clot took up the stain(s) and interfered with detail of underlying cells, nevertheless cells growing outside the clot were defined clearly. Staining of the gelatin film occurred which interfered similarly with examination of stained preparations(17). This difficulty was eliminated for the most part by removing the fragment and cell sheath with celloidin membrane(18). There was some staining in some areas of the celloidin membrane presumably due to adherence of a small amount of gelatin film material, but it did not interfere with observation of cellular detail.

Results. Similarities in growth characteristics of skin with plasma clot and on gelatin film. Primary outgrowth occurred in 76 of 77 specimens set up in plasma clot and cultured in EHu_{20} or $\text{EC}_5 \text{Hu}_{10}$ medium and from 98 of 102 fragments cultured under gelatin film in the 3 media. Initial outgrowth by either technic in the 3 media usually consisted of a single zone of compact epithelial-like cells which appeared in 2-10 days in plasma clot and in 5-14 days in gelatin film cultures. Complete or almost complete zones of epithelioid cells were present on the average, in about 9 days in the plasma clot series and in about 13 days on gelatin film. The cells were small, compact, with relatively uniform nuclei (Fig. 2 and 3). Stained preparations revealed relatively few mitotic figures which is in accord with earlier observa-

tions by Hsu(5). In some cultures, filamentous-like processes occurred in the zone of polygonal cells and also cell forms which, morphologically, were neither epithelioid nor fibroblast-like. The latter included round forms which were seen within the 1st week in plasma clot and from the 2nd to 5th week in the gelatin film group. They did not seem to increase but disappeared eventually or were obscured by the epithelioid cells. Also, oval to pear shaped forms appeared during the 2nd or 3rd week, and, at about the same time, large, elongated and fusiform cells, often with protoplasmic processes. Degeneration occurred in the zone of epithelioid cells after initial outgrowth, usually between the 2nd and 3rd week in plasma clot and in the 3rd to 4th week on gelatin film.

Differences in growth characteristics of skin with plasma clot and on gelatin film. The most striking difference that was observed between plasma clot and gelatin film technics for cultivation of adult human skin was the development of fibroblast-like cells (Fig. 4) in the majority by the former, but not any by the latter method when EHu_{20} or $\text{EC}_5 \text{Hu}_{10}$ media were employed. However, 10 of 46 cultures which grew on gelatin film in EHu_{15} developed fibroblast-like cells, particularly in those cultures in which comparatively little epithelioid outgrowth occurred. One culture consisted of pure fibroblast-like cells which were first noted on the 14th day. These differences are summarized in Table I. There were also noted in about a fourth of the plasma clot cultures areas of larger polygonal cells in the zone of uniform epithelioid cells (Fig. 5) within 2 or 3 weeks and also in some preparations, usually within a week an amorphous ring-like membrane

TABLE I. Relationship between Development of Epithelioid and Fibroblast-Like Cells from Adult Human Skin Cultured in Plasma Clot and under Gelatin Film.

Medium	Substrate	No. specimens cultured	No. with growth	No. with epithelioid growth	Time epithelioid-like cells appeared, days	No. with fibroblast-like growth	Time fibroblast-like cells appeared, days
EHu_{20} & $\text{EC}_5 \text{Hu}_{10}$	Plasma clot*	62	61	61	2-10	42	8-22
<i>Idem</i>	Gelatin film†	44	42	42	6-14	0	
EC_{15}	" "	48	46	45	5-14	10	10-25

* Plasma clot series cultivated for 3-4 wk.

† Gelatin film series cultivated for 4-6 wk.

surrounding a portion, or all of the fragment. Large polygonal cells of this type were observed in only 2 of the 102 gelatin film cultures and the membrane structure was not seen in any of these preparations.

Outgrowth from fragments after subculture. Forty fragments of adult human skin were removed from the surrounding zones of cellular outgrowth and subcultured either in plasma clot or with gelatin film in the same medium employed for primary cultivation. Growth occurred from 23 of 26 fragments when subcultures were made from plasma clot to plasma clot, from plasma clot to gelatin film and from gelatin film to plasma clot. The outgrowth consisted only, or predominantly, of fibroblast-like cells from 20 of the subcultures, despite the fact that primary outgrowth was entirely or predominantly epithelioid at time of subculture (3-6 weeks). When subcultures were made with 14 fragments grown on gelatin film to the same substrate, outgrowth occurred from only 3 fragments (2 with fibroblast-like and 1 with epithelioid-like outgrowth).

Cultivation of adult oral mucosa. Twenty oral mucosa specimens from 14 donors were cultured in plasma clot with EH₂₀ or EC₅ Hu₁₀ media and 49 from 21 donors on gelatin film with the 3 media. All of these cultures were held at least 3-4 weeks and a few as long as 10 weeks. Primary outgrowth occurred from 15 specimens in plasma clot and from 34 specimens cultured on gelatin film. The initial outgrowth, in all instances, was epithelioid and similar to that from human skin (Fig. 6). However, certain significant differences were noted. (1) Wider zones of epithelioid cells occurred in some cultures of oral mucosa than in any of the skin cultures, with no obvious degenerative changes even after 9 weeks. (2) Fibroblast-like cells developed from only 5 of 49 cultures which showed growth. These were seen in about 2 weeks in a plasma clot culture and from 5-8 weeks in 4 gelatin film cultures. (3) Attachment of fragments and subsequent epithelioid outgrowth occurred on glass equally as well as on gelatin film with glass-gelatin film interfaces.

Summary. Zones of epithelioid cells from

single fragments of adult human skin and oral mucosa develop on gelatin film. Although the initial appearance of these cells was somewhat slower on this substrate, their subsequent migration or growth appeared to be comparable to that in plasma clot. Proliferation of fibroblast-like cells was observed in some skin cultures on gelatin film when the medium consisted of a modified Eagle's basal solution supplemented with 15% calf serum but not when supplemented with 20% human or 10% human and 5% calf serum. Cultural characteristics of adult human skin and oral mucosa were similar except that fibroblast-like cells were more rarely observed and extensive sheaths of epithelioid cells occurred on glass as well as on gelatin film from oral mucosa.

We wish to acknowledge the valuable suggestions and criticisms made by Dr. Bernard Appel, Dept. of Dermatology, Tufts Univ. School of Medicine.

1. Lewis, S. R., Pomerat, C. M., Ezell, D., *Anat. Rec.*, 1949, v104, 487.
2. Parshley, M. S., Simms, H. S., *Am. J. Anat.*, 1950, v86, 163.
3. Ulloa-Gregori, Q., Blocker, T. C., Nowinski, W. W., Pomerat, C. M., *Tex. Rep. Biol. and Med.*, 1950, v8, 400.
4. Bassett, C. A., Evans, V. J., Earle, W. R., *Plast. and Reconstruct. Surg.*, 1956, v17, 421.
5. Hsu, T. C., *Tex. Rep. Biol. and Med.*, 1952, v10, 336.
6. Kepes, J., Georgiade, N., Eiring, A., Pickrell, K., *Plast. and Reconstruct. Surg.*, 1958, v21, 483.
7. Puck, T. T., Cieciora, S. J., Fisher, H. W., *J. Exp. Med.*, 1957, v106, 145.
8. Pinkus, H., *Arch. f. Derm. Syph.*, 1932, v165, 53.
9. Evans, V. J., Earle, W. R., *J. Nat. Cancer Inst.*, 1947, v8, 103.
10. Bang, F. B., Gey, G. O., *Johns Hopkins Hosp. Bull.*, 1952, v91, 427.
11. Puck, T. T., Editor, Fisher, H. W., Ham, R. G., Marcus, P. I., Webb, N. C., *Quantitative Mammalian Cell Culture*. A summary of methods used in Dept. of Biophysics, Univ. of Colorado Med. Center, 1959.
12. Ehrmann, R. L., Gey, G. O., *J. Nat. Cancer Inst.*, 1956, v16, 1375.
13. Enders, J. F., personal communication.
14. Chang, R. S., *Cellular Biol., Nucleic Acids, and Viruses*, 1957, v5, 315.
15. Morgan, J. F., Parker, R., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, v71, 665.

16. Fisher, A., *Science*, 1949, v109, 611.
17. Weinmann, J. P., Correll, J. T., *Oral Surg., Oral Med. and Oral Path.*, 1951, v4, 891.
18. Cheatham, W. J., from Enders, J. F., and

Peebles, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1954, v86, 277.

Received December 22, 1960. P.S.E.B.M., 1961, v106.

Cardiovascular Action of Adenosine and Other Nucleosides.* (26467)

E. T. ANGELAKOS[†] AND P. M. GLASSMAN (Introduced by E. R. Loew)

Department of Physiology, Boston University School of Medicine, Boston, Mass.

Several studies have been reported on the cardiovascular actions of adenosine and other purine and pyrimidine nucleosides(1-5), some of which exert a positive inotropic effect in isolated preparations(6-10). Similarly a negative chronotropic effect and a general vasodilator action have been found under a variety of experimental conditions(1,5,11). Due to the key biochemical role that some of these compounds may play in smooth and cardiac muscle energetics, it was desirable to test in the same preparation, *in vivo*, the inotropic, chronotropic and hypotensive activities of some of these compounds, to obtain quantitative data on specificity and potency.

Materials and methods. Mongrel dogs of either sex weighing from 7.8 to 14.2 kg were anesthetized with intraperitoneal injections of sodium pentobarbital. Blood pressures were measured through an Hg manometer. Myocardial contractility was measured with a Walton strain gauge arch(12) sutured on the left ventricular myocardium. Records of myocardial tension and ECG were obtained on a 2-channel Sanborn recorder. All injections were made intravenously into the jugular vein over a 10 minute period. Solutions were freshly prepared from the powdered compounds in 50 ml of normal saline. The list of compounds tested included: Adenosine, guanosine, inosine, cytidine, thymidine, uridine, adenine sulfate and guanine sulfate. All compounds were purchased from Nutritional Biochemicals, New York. Except as otherwise indicated, all animals were

given 1 mg/kg of atropine at beginning of each experiment.

Results. The following compounds produced no detectable cardiovascular actions in the doses indicated: Adenine (2-10 mg/kg), guanine (2-10 mg/kg), guanosine (2-25 mg/kg), inosine (2-100 mg/kg), cytidine (2-5 mg/kg), thymidine (2-50 mg/kg), and uridine (2-50 mg/kg). These were tested in 2 to 4 animals each. In larger doses (25 mg/kg) cytidine produced moderate hypotension and negative chronotropic and inotropic effects, but such doses were considered too large to deserve further study.

Of all the compounds tested only adenosine exhibited cardiovascular actions of a sufficient magnitude and consistency to warrant further study.

Quantitative results on the inotropic, chronotropic and hypotensive effects of adenosine were obtained at several dose levels (0.1 to 2 mg/kg) from a total of 113 injections in 10 dogs (Fig. 1). Doses larger than 2 mg/kg, up to 25 mg/kg, produced no greater effects. In all instances recovery to control levels was attained within a few seconds (Fig. 2). Since in all of the above mentioned experiments the animals were fully atropinized, the observed actions of adenosine can not be classified as cholinergic in spite of the apparent similarity to the cardiovascular actions of acetylcholine. In these animals atropine was used to eliminate the reflex vagal effects on heart rate associated with hypotension.

Since there have been reports that atropine and vagotomy may abolish some of the cardiovascular actions of adenosine, experiments were designed to test this point using

* Supported by a grant from Massachusetts Heart Assn.

[†] USPHS Senior Research Fellow.

non-atropinized animals. In repeated tests neither vagotomy nor atropine were found to interfere with the cardiovascular actions of adenosine.

Discussion. Studies with isolated heart and heart-lung preparations suggested that some of the nucleosides may have a positive while others have a negative inotropic effect (6-10). The *in vivo* studies reported here are not in general agreement with this concept since, with the exception of adenosine, most of the nucleosides tested had no significant cardiac actions. The effects of adenosine in isolated preparations are similar to those reported herein, however the positive inotropic actions found among certain nucleosides (guanosine, uridine, thymidine, inosine) (8,9) must be associated with some conditions which obtain only in isolated preparations. It is also possible that this activity is manifested only in failing preparations under special conditions. In this connection it is noteworthy that digitalis produces a positive inotropic effect when contractility is measured with a cardiac strain gauge arch *in vivo* (13).

Recently Thorp and Cobbin(5) have reported that 2-chloro-adenosine is more active than adenosine at least regarding its vasodilator potency. Furthermore this compound is said to have a longer duration of action. These authors report that atropine blocks the negative chronotropic effects of 2-chloro-adenosine which is at variance with our findings with adenosine. However our results are in agreement with studies on the effect of

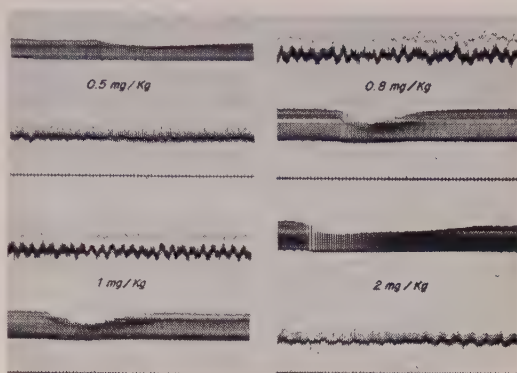


FIG. 2. Records of adenosine effects on contractility and heart rate (time markers 0.1 sec.).

adenosine, acetylcholine and atropine on atrial resting and action potentials by Johnson & McKinnon(10).

Summary. The cardiovascular effects of adenosine and other nucleosides were studied in dogs. Adenosine produced hypotension and negative chronotropic and inotropic effects of short duration. Quantitative dose-effect relationships were obtained in each case. Other nucleosides tested exhibited no significant cardiovascular actions *in vivo*.

1. Sydow, V., Ahlquist, R. P., *J. Am. Pharm. Assn., Sci. Ed.*, 1954, v43, 166.
2. Drury, A. N., *Physiol. Rev.*, 1936, v16, 292.
3. Somlo, E., *Lancet*, 1955, v268, 1125.
4. Rand, M., Stafford, A., Thorp, R. H., *Aust. J. Exp. Biol. Med. Sci.*, 1955, v33, 663.
5. Thorp, R. H., Cobbin, L. B., *Arch. internat. pharmacodyn.*, 1959, v118, 95.
6. Schenberg, S., *Acta physiol. lat amer.*, 1956, v6, 137.
7. Hollander, P. B., Webb, J. L., *Circul. Res.*, 1957, v5, 349.
8. Cook, M. H., Greene, E. A., Lorber, V., *ibid.*, 1958, v6, 735.
9. Buckley, N. M., Tsuboi, K. K., Zeig, N. J., *ibid.*, 1959, v7, 847.
10. Johnson, E. A., McKinnon, M. G., *Nature*, 1956, v178, 1174.
11. Sherrod, L., *J. Pharm. Exp. Ther.*, 1950, v98, 29.
12. Boniface, K. J., Brodie, O. J., Walton, R. P., *PROC. SOC. EXP. BIOL AND MED.*, 1953, v84, 263.
13. Walton, R. P., Leary, J. S., Jones, H. R., *J. Pharm. Exp. Ther.*, 1950, v98, 346.

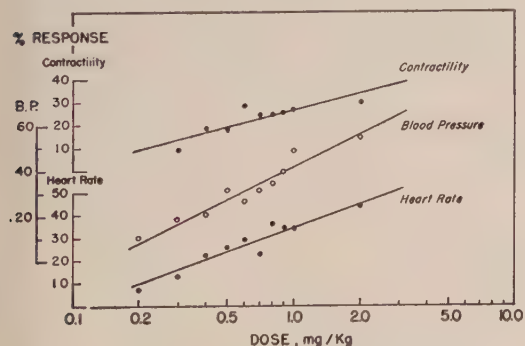


FIG. 1. % decrease in blood pressure, cardiac contractility and heart rate with submaximal doses of adenosine.

Received February 1, 1961. P.S.E.R.M., 1961, v106.

Liver Ketogenesis. IV. Ketogenesis in Thiamine Deficient Rats and Effect of Hydrolyzed Glucose Cycloacetoacetate Feeding.* (26468)

J. M. KHANADE AND M. C. NATH

Dept. of Biochemistry, Nagpur University, Nagpur, India

Thiamin deficiency is known to cause disturbances in carbohydrate metabolism such as hyperglycemia, reduced sugar tolerance and rise in blood pyruvate(1,2). The authors have reported that glucose cycloacetoacetate (GCA) a crystalline product obtained by condensation of glucose with acetoacetate, increases pyruvate utilization by rat liver slices and diaphragm *in vitro*(3). GCA (hydrolyzed) was also found to be antiketogenic *in vitro* and *in vivo*(4). In view of these findings, our study of the effect of hydrolyzed GCA or GCA (h) feeding on pyruvemia in B₁ deficiency is reported here. Liver ketogenesis and acetoacetate utilization by kidney slices and diaphragm of rats given B₁ deficient diet for a period of 4 weeks were studied. The effect of GCA (h) feeding along with deficient diet is also reported.

Method. Male albino rats growing steadily on standard diet were divided into 4 groups. Group I was given normal diet along with thiamin. Group II was fed B₁ deficient diet, the composition of which is given below. Group III received .8% GCA (h) (of diet by wt.) along with deficient diet. Because B₁ deficient rats are known to eat less, Group IV, which received the measured amount of diet equivalent to that consumed by Group II, was included.

Composition of diet	%
Starch	68
Vitamin free casein	20
Groundnut Oil	5
Salt Mixture	4
Cod liver oil	2

Vitamin mixture added mg/kg of above diet every day: Riboflavin, 4, Pyridoxine 2, Nicotinic acid 25, Cal. pantothenate 20, choline chloride 1000, Biotin .1, Folic Acid 2, Vit. B₁₂ .03, Vit. K 10, Vit. E 25. Thiamin,

50 mg/kg was added to the diet fed to Groups I and IV.

GCA was prepared by the method given by West(5) as modified by Nath *et al.*(6). It was hydrolyzed in 2N HCl for 12 min in boiling water bath. Other details have been published(3). At the end of 4th week feeding period, when rats were expected to develop B₁ deficiency as confirmed by steady weight loss and reduced food consumption, they were sacrificed by decapitation. The following estimations were made: Blood sugar by Hagedorn and Jensen method(7), blood pyruvate by Friedman(8) and liver glycogen by that of Good *et al.*(9). Wet weight of total liver, kidneys and adrenals was recorded. Tissue preparation for *in vitro* work was done by the method of Umbreit *et al.*(10). Liver and kidney slices were cut free-hand using razor blade and suspended in 3 ml of Krebs Ringer phosphate buffer, pH 7.2 with appropriate substrate. Incubation was carried out in conventional Warburg vessels for 2 hours at 37.5°C with constant shaking. At the end of incubation period, acetoacetate in media was estimated by menometric method of Edson(11).

Results and discussion. Hyperglycemia and pyruvemia in B₁ deficient rats (Group II) as compared to normals as well as controls (Group IV) are shown in Table I. GCA (h) feeding to B₁ deficient rats restores these values to normal. Our previous finding(3) that GCA (h) increases pyruvate utilization *in vitro* is thus substantiated. Thiamine is reported to be essential for lipogenesis(12). Liver glycogen and lipids are significantly diminished in B₁ deficiency (Group II) but only slightly in Group IV, *i.e.*, rats receiving restricted amount of diet. This indicates that besides inanition, B₁ deficiency has depressing effect on liver glycogen and lipid content. We have reported earlier that GCA (h) increases glycogen synthesis in liver slices and diaphragm(13). Chronic under-nutrition ex-

*The authors record their thanks to the Council of Scientific & Industrial Research for a Research Grant.

TABLE I. Blood Sugar, Blood Pyruvate, Liver Glycogen and Lipids, % of Tissues Weights (Wet Wt) and Acetoacetate Production by Liver Slices and Its Utilization by Diaphragm and Kidney Slices.

Group	I	II	III	IV
No. of rats	8	12	8	6
Blood				
Sugar, mg/100 cc	90 \pm 3	105 \pm 3	91 \pm 4	92 \pm 4
Pyruvate, mg/100 cc	.86 \pm .12	5.0 \pm .3	1.13 \pm .2	.91 \pm .16
Liver				
Glycogen, g/100 g wet wt of tissue	3.3 \pm .16	2.0 \pm .18	2.8 \pm .21	3.1 \pm .2
Lipids, <i>idem</i>	4.8 \pm .5	3.4 \pm .3	4.4 \pm .3	4.2 \pm .4
Tissue wt				
Liver, g/100 g wet wt of tissue	3.5 \pm .1	4.3 \pm .13	4.0 \pm .1	3.8 \pm .15
Kidney, <i>idem</i>	.23 \pm .03	1.16 \pm .05	.8 \pm .04	.81 \pm .05
Adrenals, mg/100 g wet wt tissue	12 \pm 1	18 \pm 2.5	11.8 \pm 2	14 \pm 2
Acetoacetate produced,* μ l/100 mg of liver slices				
Butyrate, .017 M	66 \pm 5	100 \pm 8	69 \pm 6	73 \pm 7
Acetate, .08 M	50 \pm 4	80 \pm 7	55 \pm 5	60 \pm 6
Acetoacetate utilized† in μ l/100 mg wet wt of tissue				
Kidney slices	68 \pm 5	51 \pm 4	69 \pm 6	58 \pm 5
Diaphragm	45 \pm 3	15 \pm 4	40 \pm 5	35 \pm 6

* μ l CO₂ produced after decarboxylation of acetoacetate with aniline citrate.† 150 μ l acetoacetate added as substrate.

Krebs' Ringer phosphate buffer, pH 7.2, temp. 37.5°C. Incubation period 2 hr. Air as gas phase.

erts a non-specific stress and causes hypertrophy of adrenals(14) but this hypertrophy is also present in B₁ deficiency even when evaluated with pair feeding technic(15). Hypertrophy of tissues, especially of adrenals, in B₁ deficiency is also shown in our results (Table I). GCA (h) feeding has been found to maintain normal adrenal weight in deficient animals.

Acetoacetate production by liver slices is considerably increased in B₁ deficiency and its utilization by diaphragm and kidney slices is decreased. Liver glycogen has been known to influence lipogenesis and ketogenesis(16). Mirsky(17) has shown that depletion of liver glycogen causes greater oxidation of fatty acids and greater production of ketone bodies. It may be assumed that lower levels of liver glycogen in B₁ deficient rats coupled with decreased lipogenesis by adipose tissue, accelerate ketogenesis by liver slices. GCA (h) feeding increases acetoacetate utilization and prevents its excessive production.

Beatty *et al.*(18) have reported that glucose has no effect on acetoacetate utilization in the muscle and diaphragm *in vitro* but GCA, which on hydrolysis gives rise to a

enediol glucose(19) has great effect in accelerating acetoacetate utilization.

Summary. B₁ deficiency in rats causes hyperglycemia, pyruvemia and reduces liver glycogen and total lipids. Marked hypertrophy of tissues especially of adrenals is also found. Acetoacetate production by liver slices is increased and its utilization by kidney slices and diaphragm decreased. Beneficial effects of GCA (h) feeding to B₁ deficient rats is evident from the results presented.

1. Sato, K., Kaiho, N., Tohoku, *J. Exp. Med.*, 1949, v510, 218.
2. Shujatali, Pak., *J. Sci. Res.*, 1949, v1, 15.
3. Nath, M. C., Khanade, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1958, v97, 706.
4. ———, *ibid.*, 1960, v103, 559.
5. West, E. S., *J. Biol. Chem.*, 1927, v74, 561.
6. Nath, M. C., Chitale, R. P., Bhavani, B., *Nature*, 1952, v170, 545.
7. Hagedorn, H. C., Jensen, B. N., *Biochem. Z.*, 1923, v135, 46.
8. Friedman, T. E., Haugen, G. E., *J. Biol. Chem.*, 1943, v147, 415.
9. Good, C. A., Kramer, H., Somogyi, M., *ibid.*, 1933, v100, 485.
10. Umbreit, W. W., Burris, R. S., Stauffer, J. F.,

Manometric Technique of Tissue Metabolism, Minneapolis, Burgess, 1945.

11. Edson, N. L., *Biochem. J.*, 1935, v29, 2082.
12. McHenry, E. W., Gavin, G., *J. Biol. Chem.*, 1939, v128, 45.
13. Nath, M. C., Khanade, J. M., *J. Sci., Ind. Res.*, 1959, v18.C, 169.
14. Mulinos, M. G., Pomerants, L., *J. Nutr.*, 1941, v19, 493.
15. Deane, H. W., Shaw, J. H., *N. Nutr.*, 1947, v34, 1.

16. Shaw, W., Gurin, S., *Arch. Biochem. Biophys.*, 1953, v47, 545.

17. Mirsky, I. A., *J. Am. Med. Assn.*, 1942, v118, 690.

18. Beatty, C. H., Marco, A., Peterson, R. D., Block, R. M., West, E. S., *J. Biol. Chem.*, 1960, v235, 2774.

19. Nath, M. C., Bhattathiry, E. P. M., *Nature*, 1956, v178, 1233.

Received January 3, 1961. P.S.E.B.M., 1961, v106.

Effects of Plasma Erythropoiesis Stimulating Factor (E.S.F.) at Different Time Intervals After Single Injection. (26469)

G. HODGSON* (Introduced by H. Borsook)

Biology Division, California Institute of Technology, Pasadena

It has been suggested(1,2) that erythropoiesis stimulating factor (E.S.F) stimulates differentiation of stem cells into red cell precursors. Results of histological study of the spleen of polycythemic mice treated with E.S.F. support this view(3). More detailed analysis of several effects of E.S.F. might provide evidence for or against this theory. Therefore, amino acid and iron incorporation in hemoglobin, and reticulocyte levels were measured at various time intervals after a single intravenous injection of E.S.F.

Methods. Normal Webster Swiss female mice 24-30 g obtained from Diablo, Berkeley, Cal. were used. E.S.F. extract, No. LD11, was prepared from plasma of rabbits made severely anemic with phenylhydrazine by methods already described(4). Its activity was about 0.7 CS 1 unit/mg or 7 Co units/mg. This extract is of the same type as Extract CS 1 which was assayed in several laboratories(4).

Several criteria were employed to measure the effects of the extract. a) Measurement of C¹⁴ amino acid uptake in globin as proposed by Dovey *et al.*(5). b) Measurement of Fe⁵⁹ in plasma and spleen at 2 hours and

in red cells at 48 hours after tracer injection. c) Reticulocyte counts. d) Measurement of reticulocytes by determining *in vitro* uptake of Fe⁵⁹ and C¹⁴ leucine into washed red cells from 5 mice, by methods described by Borsook(6).

The experimental design was the following: on day 0 the mice received 0.25 ml of .9% NaCl solution containing 2 mg LD11 intravenously (tail vein). Controls received .9% NaCl solution. For the C¹⁴ amino acid experiments groups of mice—number of animals shown in Table I—were injected intravenously at 12, 24, 48, 72, and 96 hours after the extract with 1 μ C C¹⁴ uniformly labelled amino acids obtained by acid hydrolysis of C¹⁴ labelled chlorella protein (CFB6 Radiochemical center, Amersham, England) with a specific activity of 1 mc/5.6 mg. One week later, a time when C¹⁴ activity globin had levelled off, mice were exsanguinated by heart puncture under ether anesthesia. In initial experiments pooled red cells from 6 mice were washed thrice with 30 vol. of ice cold .9% NaCl; 0.10 ml of the red cell suspension was dried on planchettes using the technique described by Lowy *et al.*(7), and counted in Nuclear Chicago automatic gas flow counter. The rest of the red cells were lysed with 20 vol. of distilled water, insoluble material was centrifuged down, the clear super-

* Research Fellow supported by I.C.A. under the visiting scientists program administered by Nat. Acad. Sci. Permanent address: Inst. de Fisica y Matematicas U de Chile, Santiago.

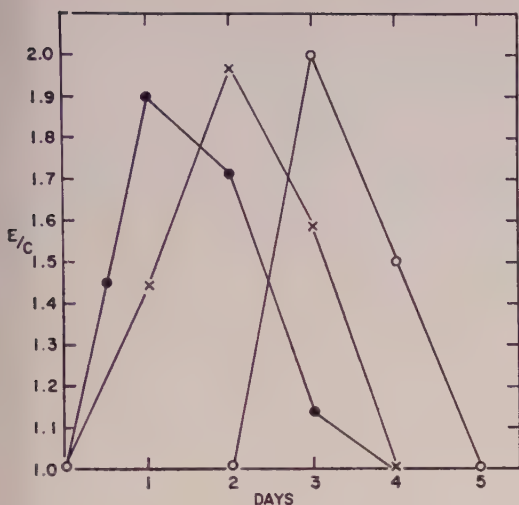


FIG. 1. Effect of E.S.F. as a function of time after intrav. inj. Ordinate: E/C = ratio of experimental to control values for ●—● C^{14} in Hb. ×—× fraction of plasma iron going to erythroid tissue per hour. ○—○ reticulocytes. Abseissa: Days after E.S.F. inj.

nantant was made 5% with respect to TCA, the precipitated proteins were washed with TCA, acid acetone, alcohol-ether, ether, dried, plated and counted. Results of both methods of counting were correlated. For 6 pools with activities ranging from 60-375 cpm, the value of r was 0.985 and the slope 1. In view of this finding, C^{14} uptake into globin was measured in the experiments to be described by counting plated washed red cells. For the Fe^{59} experiments, other groups of mice were injected with Fe^{59} at 24, 48, 72, and 96 hours after injection of the extract. Two hours later, radioactivity in plasma and spleen was determined in some of the mice. The remaining mice were bled 48 hours after tracer injection and erythrocyte Fe^{59} was determined. Counting was done as in the experiments of Lowy *et al.*(7). For calculation of Fe^{59} uptake the blood volume was taken as 6.7 ml/100 g. This value was obtained by measuring the volume of distribution of Fe^{59} labelled erythrocytes.

Reticulocytes were stained using new methylene blue and the fraction of these cells was determined by counting a total of 100 reticulocytes in incubation experiments, and 1000 red cells in other experiments. Pooled red cells from blood drawn 72 hours after

injection of the extract were used for incubation experiments.

Results. Fig. 1 and Table I show that C^{14} amino acid uptake into globin was at a maximum when the tracer was injected 24 hours after the extract. Uptake was also above normal when tracer was injected 12 and 48 hours after extract, but at 72 hours it was equal to controls. Plasma iron turnover rate and uptake of iron by the spleen were slightly above control values at 24 hours, maximum at 48, slightly elevated at 72 hours, and equal to the controls at 96 hours. Fe^{59} uptake in red cells was high from 24 to 72 hours after E.S.F. administration. The product of plasma iron turnover rate and percentage of Fe^{59} appearing in the red cells at 48 hours was taken as an estimate of the fraction of plasma iron going to erythroid tissue (F.I.Er.) Values of spleen Fe^{59} and F.I.Er. are in close agreement (Table I). Fig. 1 represents the variation of F.I.Er. and reticulocyte count as a function of time after E.S.F. injection. The increased reticulocyte count manifested itself by increases in uptake of C^{14} leucine and Fe^{59} into the incubated red cells of blood drawn 72 hours after injection of the extract (Table II). Analysis of the dose response study showed that incorporation of C^{14} leucine and Fe^{59} was a function of the log dose of extract injected. The increase in C^{14} leucine and Fe^{59} uptake is proportionately greater than the increase in reticulocyte percentage. This probably reflects the fact that Hb synthesis is not only a function of reticulocyte number but also of degree of maturity. The more immature reticulocytes synthesize more Hb(8).

Discussion. The first observed effect of E.S.F. in an increased uptake of C^{14} amino acids into globin, as early as 12 hours after injection, with a maximum at 24 hours. Maximum iron uptake into Hb is at 48 hours. Reticulocytosis appears at 72 hours.

The increased uptake of C^{14} leucine and Fe^{59} by incubated erythrocytes from blood drawn 72 hours after injection of extracts represents an objective method of measuring reticulocytes. It is conceivably more precise than reticulocyte counts, which have a high

TABLE I. Effects of E.S.F. as a Function of Time after Intravenous Injection of Extract. First column indicates time interval between injection of E.S.F. and of tracer. Number in parentheses indicates number of mice in the group in which measurement was carried out.

Time, hr	1	2	3	4	5	6	7
	Retie., %	C ¹⁴ Hb, cpm	Fe ⁵⁹ , % D plasma (2 hr)	PITR, %/hr	% Fe ⁵⁹ erythrocytes (at 48 hr)	F.I.Er, %/hr	% Fe ⁵⁹ spleen (2 hr)
0	2.05 ±.15(10)	790 ± 41.5 (14)	24.5 ±1.24 (13)	70	46.8 ± 4.08(10)	32.9	3.8 ±.60(10)
12	—	1160 ± 60.0 (7)	—	—	—	—	—
24	—	1500 ± 71.0 (14)	20.5 ± .63 (6)	79.5	59.6 ± 2.45 (6)	47.4	4.8 ±.69 (5)
48	2.00 ±.46 (5)	1360 ± 71.0 (14)	13.5 ± .69 (10)	100	64.8 ± 1.96 (6)	64.8	7.6 ±.95 (5)
72	4.00 ±.33 (5)	850 ±102.0 (6)	20.3 ± .62 (9)	80	65.5 ± 2.95 (6)	52.4	5.9 ±.91 (5)
96	3.05 ±.42 (6)	810 ± 77.0 (5)	24.3 ±1.43 (5)	70	—	—	3.6 ±.52 (5)
120	1.93 ±.15 (6)	—	—	—	—	—	—

PITR = plasma iron turnover rate, fraction of plasma iron turned over per hr (%/hr) = $\frac{1}{2} \ln \frac{1}{\text{plasma Fe}^{59}}$ where plasma Fe⁵⁹ = fraction of inj. Fe⁵⁹ remaining in plasma at 2 hr. F.I.Er = fraction of plasma iron going to erythroid tissue = PITR × % Fe⁵⁹ in erythrocytes at 48 hr.

standard deviation, especially at low levels (9).

The experimental findings can be explained by assuming that initially, under the influence of E.S.F., a greater number of pro- and basophilic erythroblasts appear in the marrow(3). The presence of more cells of this type manifests itself early (12 hours) by an increased uptake of amino acids into globin, at a rate greater than the uptake of iron into heme. Hammarsten *et al.*(10) have shown that these early cells synthesize globin faster than heme, and autoradiographic studies(11) indicate that they take up 4 times more amino acids than polychromatic normoblasts and 60 times more than orthochromic normoblasts. Some time later (48 hours) when the newly formed cells have matured to the polychromatic stage, heme synthesis is at a maximum as indicated by histochemical studies (12) and by autoradiographic Fe⁵⁹ determinations in rat marrow(13). At this period, 48 hours after E.S.F. injection when plasma iron turnover and Fe⁵⁹ incorporation into Hb is at its maximum, C¹⁴ amino acid uptake

is still high, for although individual polychromatic normoblasts take up less amino acid than the earlier forms(11), more cells of this type are present. Later, 72 hours after E.S.F. injection, the cells have matured to the reticulocyte stage in which, as the incubation studies show, terminal globin and heme synthesis go on at approximately the same rates(6).

In conclusion, these data can be tentatively correlated with those of Filmanowicz and Gurney(3) in the polycythemic mouse spleen, by interpreting the early increase in C¹⁴ amino acid uptake into globin as a reflection of an increase in the early forms of erythroid cells 24 hours after E.S.F. injection and the increase in Fe⁵⁹ uptake at 48 hours as reflecting maturation of these cells to normoblasts. The reticulocyte peak at 72 hours seen in normal mice coincides with the maximum reticulocytosis observed in polycythemic mice. Taken together, these data indicate that the sequence and timing of the events are similar in normal and physiologically depressed erythropoietic tissue.

Summary. Several effects of E.S.F. in normal mice were studied as a function of time after injection. The first change was a marked increase of C¹⁴ amino acid uptake by globin at 24 hours. This was followed at 48 hours by increase in iron consumption by erythroid tissue, and at 72 hours by an increase of reticulocyte count in peripheral blood as well as by increase in Fe⁵⁹ and leucine C¹⁴ uptake

TABLE II. Leucine C¹⁴ and Fe⁶⁰ Uptake by Ineubated Erythrocytes from Blood Drawn 72 Hr after E.S.F. Injection in Female Webster Swiss Mice.

E.S.F. inj.	Retie., %	C ¹⁴ , cpm/.1 ml	Fe ⁶⁰ , cpm/.1 ml
Control	1.65	61	740
.25 mg	3.35	169	2483
.50 "	4.4	219	3031
1.5 "	4.4	312	3766

by incubated red cells.

1. Alpen, E. L., 1959, in *The kinetics of cellular proliferation* (F. Stohlman, ed.) p. 299. Grune and Stratton, New York.
2. Erslev, A. J., *Blood*, 1959, v14, 386.
3. Filmanowicz, E., Gurney, C. W., unpublished data cited by White, W. F., Gurney, C., Goldwasser, E., Jacobson, L. O., *Recent Progress Hormone Research*. 1960, p219. Academic Press Inc., New York.
4. Keighley G., Lowy, P., Borsook, H., Goldwasser, E., Gordon, A. S., Prentice, T., Rambach, W., Stohlman, F., Van Dyke, D., *Blood*, 1960, v16, 1424.
5. Dovey, A., Holloway, R., Piha, R. S., Humphrey, J. H., McFarlane, A. S., Radio Isotope Conf. 1954, vI, p337 (J. E. Johnston, ed.) Academic Press, New York.
6. Borsook, H., Conference on Hemoglobin, Nat.

Acad. Sci., 1958. publication 557, 111.

7. Lowy, P. H., Keighley, G., Borsook, H., Graybiel, A., *Blood*, 1959, v14, 262.
8. Holloway, B., Ripley, S. H., *J. Biol. Chem.*, 1952, v196, 695.
9. Dacie, J. V., *Practical Hematology*, 1950, Churchill Ed., London.
10. Hammarsten, E., Thorrell, B., Aquist, S., Eliasson, W., Berman, L. A., *Exp. Cell Res.*, 1953, v5, 404.
11. Pleri, A., Mariana, G., Gavosto, F., *Exp. Cell Res.*, 1960, v20, 645.
12. Thorrell, B., *Acta. Med. Scand.*, 1947, Suppl. 200.
13. Harris, E. B., *Advances in Radiobiology*, 1956, p333 (DeHevesy Forsberg and Abbot, ed) Oliver and Boyd, London.

Received January 4, 1961. P.S.E.B.M., 1961, v106.

Rate of Localization of Anti-Rat Lung Antibody.*† (26470)

ITSURO TAMANOI, YASUO YAGI AND DAVID PRESSMAN

Department of Biochemistry Research, Roswell Park Memorial Institute, Buffalo, N. Y.

Antibody prepared in rabbits against rat lung tissue has been shown to be fixed in the lung and other organs when injected intravenously into rats(1). A similar situation exists with antibody against rat kidney in that when it is injected intravenously it localizes in the kidney with some cross-localization elsewhere(2,3). Rate of localization in kidney has been determined for the latter case(4) and it was found that for the most part the antibody is removed from the circulation as it passes through that organ. There is a second component which appears to be fixed at a somewhat slower rate. In the experiments reported here, rate of localization of anti-lung antibody in the lung was determined.

Preliminary results with the globulin fraction of anti-lung serum indicated that the lung localizing activity was too low to permit very accurate measurement in view of the relatively high background localization in the lung. Therefore, the study was made pri-

marily with antibodies in which the lung localizing activity had been concentrated and purified by adsorption and elution from lung tissue. The major part of the kidney localizing activity was removed from the purified preparation by treatment with kidney sediment.

Materials and methods. Sprague-Dawley rats were used throughout the studies. *Sera.* Anti-rat lung sera were obtained by injecting perfused rat lung homogenate intraperitoneally into 8 rabbits 3 times a week for 4 weeks and bleeding one week after last injection. The dose given was 0.5 g wet tissue per injection. The γ -globulin fraction of each antiserum (γ G Anti-R Lu) and of normal rabbit serum (γ GNS) (Pentex, Inc.) was prepared by ethanol fractionation(5). The γ G anti-R Lu from a single rabbit showing the highest lung localizing activity relative to localization elsewhere was selected for this study. The proteins were radioiodinated as described previously(6,7). *Preparation of sediments:* Frozen rat lungs previously perfused with saline were minced with scissors and homogenized with 4.5 vol. physiological saline

* Supported by grant from Nat. Heart Inst.

† Presented at 44th Annual Meeting of Am. Assn. of Immunol. April, 1960, Chicago, Ill.

and 0.5 vol. borate buffer, pH 8.0, by means of a glass tissue grinder. The homogenate was centrifuged at 1,500 RCF for 15 min. at 5°C and the sediment removed. The supernatant was recentrifuged at 25,000 RCF for 15 min. and the collected sediment was washed several times, then lyophilized. This preparation of lung high speed sediment, referred to as Lu H Sed was found to concentrate lung localizing antibody more specifically than the low speed sediment. Rat kidney sediment (K L Sed) was collected by centrifuging the homogenate at 1,500 RCF, washed and lyophilized. *Preparation of fibrin:* Fibrinogen was prepared from normal rat plasma by fractionation with phosphate buffer similar to that for human fibrinogen(8). Fibrinogen (30 mg) was dissolved in borate buffer, pH 8.0 and mixed with 20 NIH units of bovine thrombin (1.0 mg lyophilized bovine thrombin, Parke, Davis & Co.). After standing at 37°C for 1 hour, fibrin was collected on a stirring rod, chopped in small pieces with scissors and homogenized with a glass tissue grinder to make a fine suspension. After centrifuging and washing, the fibrin was used for adsorption. *Purification of anti-lung antibody:* 16 mg of radiolabeled γ -globulin fraction of anti-lung serum was treated with 16 mg of Lu H Sed in the presence of 0.2 ml of normal rabbit serum at 37°C for 1 hour. The sediment was collected by centrifuging the mixture at 25,000 RCF for 15 min. and washed twice with 10 ml of borate buffer pH 8. The sediment was suspended in 5 ml borate buffer and treated at 60°C for 20 min. to elute the adsorbed antibody. The same procedure was repeated with the residue using 3 ml borate buffer. Both eluates were combined. Of the original radioactivity, 4.5% was recovered in the eluate. The purified anti-lung antibody (eluate) was further treated once with rat fibrin prepared from 30 mg fibrinogen, then twice with 16 mg of K L Sed to remove anti-fibrin(9) and the major part of kidney localizing antibody. The final supernatant was used for injection into donor animals. The final recovery was 3.2% of the original radioactivity. Radioiodinated normal serum globulin was treated in the same

manner. Recovery of radioglobulin in the eluate was 0.91%. After adsorption with fibrin and kidney sediment, 0.72% of the original radioactivity was found in the final supernatant. This was used for injection into donors of control groups.

Results. The purified anti-lung antibody described above was diluted with saline containing 2% of normal rat serum, and 2 ml portions, containing 34 μ g radio-globulin, were injected intravenously (tail vein) into a series of 14 Sprague-Dawley rats weighing 130-150 g. After a certain period of time blood from a group of 2 donor rats was taken from the abdominal aorta with heparinized syringes and collected plasmas were pooled. The localizing activity remaining in the plasma from each group was determined by injecting 0.5 ml of the plasma (diluted to 2.0 ml with borate buffer), into each of 5 recipient rats. The initial localizing activity of the antibody preparation (zero circulation time) was determined without passive transfer by direct injection of the original material (diluted similarly) into a group of recipient rats. All recipient rats were perfused 3 days after injection, the tissues removed(7) and radioactivity was determined as described previously(6).

A similar preparation from normal serum globulin (11.5 μ g/donor rat) was used for donor rats of control groups and the passive transfer carried out as mentioned above.

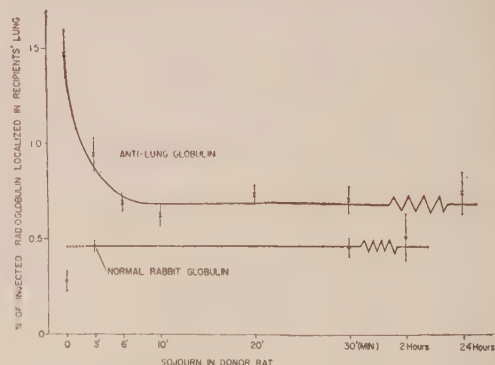


FIG. 1. Disappearance of lung localizing activity of anti-lung antibody from circulation. Purified anti-lung antibody and the similar preparation from normal serum globulin (control) were used. Abseissa: circulation time in donor rats. Ordinate: % of inj. radioglobulin, remaining in donor plasmas, which was fixed by the lungs of assay rats.

TABLE I. Disappearance of Localizing Activity from Circulation (Anti-Lung Antibody). Donor rats were inj. with purified anti-lung antibody and bled at various times after inj. Localizing activity of radioglobulin remaining in circulation at time of bleeding was assayed by inj. of the plasma into 5 assay rats. (3 day assay.)

Time between inj. & bleeding	% of inj. globulin remaining in donor's plasma*	Localization of remaining antibody in assay rats (% dose in total organ)					Net localization of remaining antibody† (% dose in total organ)			
		Lung	Kidney	Liver	Spleen	Blood*	Lung	Kidney	Liver	Spleen
0 min.‡	—	1.47	.48	2.58	.41	12.2	1.04	.36	2.10	.34
3	84.1	.94	.54	2.11	.38	15.0	.51	.42	1.63	.31
6	67.1	.69	.42	1.26	.28	18.1	.26	.30	.78	.21
10	59.6	.62	.32	1.08	.26	18.7	.19	.20	.60	.19
20	51.2	.73	.42	1.18	.24	18.9	.30	.30	.70	.17
30	47.8	.70	.22	.75	.15	15.6	.27	.10	.27	.08
24 hr	18.5	.74	.20	.74	.14	28.2	.31	.08	.26	.07

* Calculated assuming total blood as $\frac{1}{10}$ of body wt.

† Localization corrected for nonspecific localization of control preparation from GNS (Table II).

‡ Zero time: direct inj. into 5 assay rats.

TABLE II. Disappearance of Localizing Activity from Circulation (Control Preparation from GNS). Donor rats were inj. with purified GNS and bled at various times after inj. Nonspecific localizing activity of radioglobulin remaining in circulation at time of bleeding was assayed by inj. of the plasma into 5 assay rats. (3 day assay.)

Time between injection and bleeding, min.	% of inj. globulin remaining in donor's plasma*	Localization of remaining radioglobulin in assay rats (% dose in total organ)				
		Lung	Kidney	Liver	Spleen	Blood*
0†	—	.28	.10	.45	.05	15.7
6	64.0	.46	.13	.62	.06	28.9
30	45.7	.45	.13	.47	.09	31.8
120	38.2	.51	.10	.36	.07	32.6
	Avg	.43	.12	.48	.07	

* Calculated assuming total blood as $\frac{1}{10}$ of body wt.

† Zero time: direct inj. into 5 assay rats.

Preliminary experiments indicated that the fixation of lung-localizing antibody takes place rapidly, essentially within one hour after injection. Therefore, the experiment was carried out with shorter circulation times of 3, 6, 10, 20, 30 min. and 24 hours. The results are shown in Table I and Fig. 1. Net localizing activity remaining in the donors' plasma was calculated by subtracting the average background values of control groups (Table II). The lung localizing activity of the donor plasma decreased to 50% level after only 3 minutes of circulation and to 25% level in 6 minutes, then remained at this level up to 24 hours of circulation. This indicates that the lung localizing antibody in anti-lung serum is fixed by donor rats very rapidly. Indeed, all the available lung localizing antibody seems to be cleared from the

circulation in 6 minutes. Liver, kidney, and spleen localizing antibody although still present in the purified preparation,† are also fixed rapidly, but complete clearance from the

‡ The localizing activity of the purified antibody (before adsorption with K L Sed) can be affected by treatment with sediments from various organs. K L Sed was most effective in removing the kidney localizing antibody together with some of the liver and spleen localizing antibody. This did not affect the lung localizing antibody. The sediments from liver and spleen removed parts of the liver, spleen and kidney localizing activity without affecting the lung localizing activity. The high speed sediment from kidneys and the low speed sediment from lungs removed part of the lung localizing antibody as well as part of the other localizing antibodies. The high speed sediment from lungs was most effective in removing the lung localizing antibody.

blood seems to take a longer time than the lung localizing antibody.

Discussion. For the passive transfer method to give an accurate measure of the localization of antibody in a particular tissue, the localizing antibodies should be made as specific as possible against the target tissue in question. If the lung localizing antibodies are fixed by other tissues as well as the lung itself, the disappearance rate of lung localizing activity from the donor's circulation would not indicate the true rate of fixation by lung. However, it has been shown that the anti-lung antibody once localized in the lung does not go to other organs to any appreciable extent, when eluted and reinjected(10). Therefore, we feel that the data obtained here represent a rather accurate estimate of the localization rate occurring in the lung. Thus, the lung localizing antibody seems to be fixed by lungs very rapidly and the responsible antigens must be in close contact with the circulating blood.

The data are not accurate enough to determine whether the trapping efficiency of lung is greater than that of kidney or liver for their respective antibodies. The more rapid clearance by lung may be due to the greater circulation through the lungs which get the full cardiac output as compared with the other organs which get only part.

Summary. Rate of localization of anti-rat

lung antibody in lungs was determined by a passive transfer method. The purified anti-lung antibody was injected into donor rats and the lung localizing activity remaining in the circulation was assayed by injecting the plasmas into groups of recipient rats. The lung localizing antibody was cleared from the circulation very rapidly, indicating that the responsible antigens must be in very close contact with the circulating blood.

1. Pressman, D., Eisen, H. N., *Proc. Soc. Exp. Biol. and Med.*, 1950, v73, 143.
2. Eisen, H. N., Pressman, D., *J. Immunol.*, 1950, v64, 487.
3. Pressman, D., Keighly, G., *ibid.*, 1948, v59, 141.
4. Blau, M., Day, E. D., Pressman, D., *ibid.*, 1957, v79, 330.
5. Deutsch, H. F., *Methods in Medical Research* (Year Book) 1952, v5, 284.
6. Day, E. D., Planinsek, J., Pressman, D., *J. Nat. Canc. Inst.*, 1956, v17, 517.
7. Yagi, Y., Pressman, D., *J. Immunol.*, 1958, v81, 7.
8. Biggs, R., MacFarlane, R. G., *Human Blood Coagulation and its Disorders*, 2nd ed., Charles C Thomas, Springfield, Ill., 1957. See also: Hiramoto, R., Yagi, Y., Pressman, D., *Cancer Research*, 1959, v19, 874.
9. Day, E. D., Planinsek, J., Pressman, D., *J. Nat. Canc. Inst.*, 1959, v22, 413.
10. Pressman, D., Sherman, B., *J. Immunol.*, 1951, v67, 21.

Received January 3, 1961. P.S.E.B.M., 1961, v106.

An Improved Method for Rapid Laboratory Diagnosis of Poliomyelitis. (26471)

LENORE R. PEIZER, BENJAMIN MANDEL AND DORA WEISSMAN
(Introduced by Morris Schaeffer)

*Bureau of Laboratories, Department of Health, and Public Health Research Institute,
New York City*

A common procedure for laboratory diagnosis of poliomyelitis involves first isolation of the viral agent from a stool specimen followed by serological identification of this agent. Attempts to expedite this procedure by carrying out serological identification directly on virus-containing stool extract have not been entirely successful principally be-

cause of the presence of non-specific cytotoxins in such material. Various aspects of this problem have been fully described and discussed(1,2).

It will be shown in this report that by differential centrifugation of infected stool extracts, virus can be concentrated and separated from cytotoxic material. Serological

identification can be performed directly on such viral preparations without intrusion of non-specific cellular reactions. Final results are then, in most instances, available in less than 5 days.

Materials and methods. Cell cultures: HeLa cells and monkey kidney (MK) cells were used. About 50,000 HeLa cells were seeded in tubes (13x100 mm) with 1 ml of medium consisting of 20% horse serum and 0.1% yeast extract in Hanks' solution. After 4-5 days' growth, medium was removed and the cell layer washed with Hanks' solution. MK cells were obtained from a commercial source* as primary tube cultures containing approximately 200,000 cells per tube.

Maintenance medium: For both isolation and identification of the viral agent, medium consisted of 2% chicken serum and 0.5% lactalbumin hydrolysate in Hanks' solution.

Antisera: Rabbits were immunized by repeated injections of live virus.

Preparation of stool specimens: A 20% suspension of the specimen was prepared in Hanks' solution (without bicarbonate) and centrifuged at 2,000 rpm for 10 minutes. Supernate was collected and centrifuged at 8,000 rpm for 20 minutes in the No. 40 rotor of the Model L Spinco centrifuge. Supernate was collected and sufficient gelatin (1% concentration in Hanks' solution) was added to bring the final concentration to 0.1%. As shown by Baron(3), presence of gelatin increases the efficiency of centrifugal sedimentation of small viruses. The specimen was then centrifuged at 38,000 rpm for 2 hours in the No. 40 rotor which was allowed to decelerate without braking. Supernate was decanted and discarded. Sediment was resuspended in 2 ml of maintenance medium and penicillin and streptomycin were added to a final concentration of 100 units each per ml. Three 2 ml suspensions were prepared and pooled for each specimen. This preparation (6 ml) which hereafter will be referred to as stool concentrate, was held at room temperature for 30 minutes.

Simultaneous isolation-identification test: To each of 5 one ml portions of stool concen-

trate were added respectively one ml of (a) type 1, (b) type 2, (c) type 3 antisera, (d) a pool of the 3 antisera and (e) Hanks' solution. An additional 2 ml of (e) was prepared to be used as inoculum for MK cells. Antisera were diluted 1:20 in Hanks' solution prior to use. Mixtures were held 45 minutes at room temperature, then 0.5 ml was added to each of 3 tube cultures of HeLa cells. In the case of specimen (e), which served as the isolation specimen, 3 tubes of MK cell cultures also were inoculated. Controls for the test were set up as follows: (f) Hanks' solution, (g, h, i) each of the 3 antisera, (j) a known type 1 poliovirus, (k) poliovirus plus known type 1 antiserum. One-half ml of each control was inoculated into each of 3 HeLa culture tubes. It was not essential to include controls (g, h, i) in every test after it had been ascertained that a given batch of antiserum was not toxic for HeLa cells. All cultures were then incubated for 2 hours at 37°C after which the inoculum was removed and replaced by one ml of maintenance medium. Cultures were then incubated at 37°C for final observation.

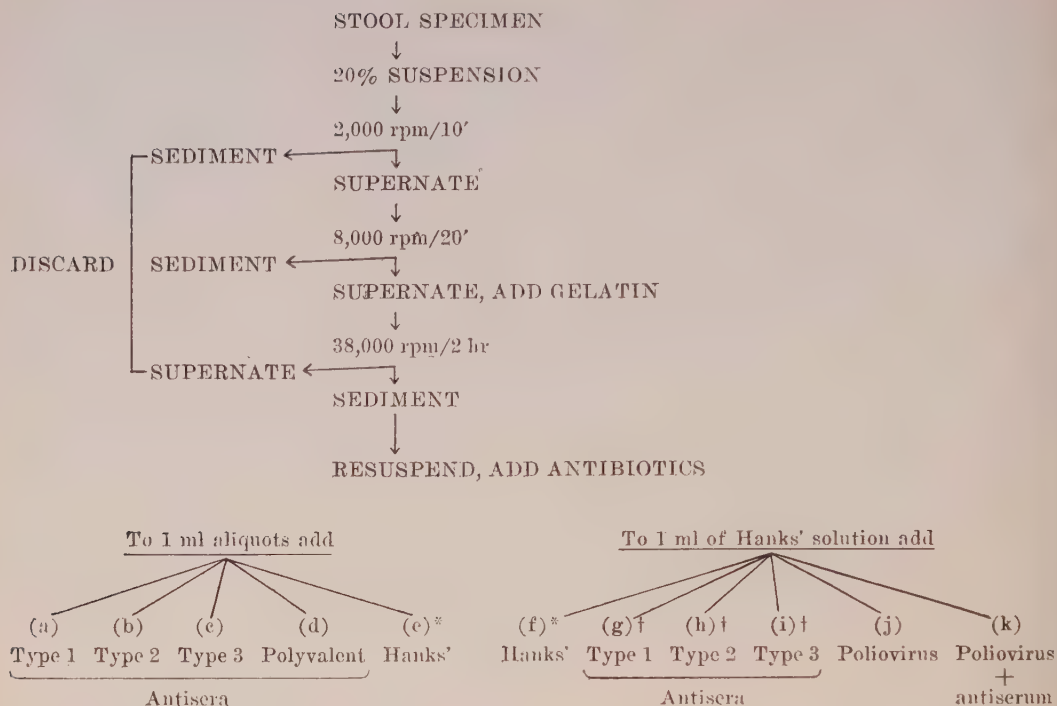
Occasionally the above routine procedure was modified as follows: Cultures were inoculated with 0.2 ml of each specimen and 0.8 ml of maintenance medium. Cultures were then incubated at 37°C for final observation without removal of inoculum.

A schematic representation of preparation of the stool specimen and protocol for the test are shown in Fig. 1.

Results. Interpretation of possible results of the test: The various possible results of this test are considered in Table I. If stool concentrate contains poliovirus type 1, 2 or 3, the reactions indicated in rows 1, 2, 3, respectively, will be observed. These results are frequently unequivocal in 2 or 3 days and in most instances final within 5 days.

If the result shown in row 4 is observed, incubation is continued for 14 days to detect either atypical poliovirus or other slow-growing enteroviruses. If after prolonged incubation cytopathic changes in isolation cultures are observed, the culture fluids are collected for further studies. If growth occurs only in MK cultures (row 5), virus is collected from

* Microbiological Assoc., Inc., Bethesda, Md.



* These are set up in double quantities to allow for inoculation of monkey kidney cultures as well as HeLa cultures.

† These controls are necessary only for untested preparations of sera; subsequently occasional inclusion either as individual or pooled sera is recommended.

FIG. 1. Preparation of stool specimen for use in simultaneous isolation-identification test.

TABLE I. Protocol of Simultaneous Isolation-Identification Test and Consideration of Possible Results.

Row No.	Neutralization				Isolation	Interpretation of results
	Stool concentrate + antiserum				Stool concentrate + Hanks'	
	1	2	3	Poly-valent	trate + Hanks'	
	(a)	(b)	(c)	(d)	(e)	
1	N*	C*	C	N	C	Poliovirus type 1 present
2	C	N	C	N	C	" " 2 "
3	C	C	N	N	C	" " 3 "
4	N	N	N	N	N—HeLa N—MK	Negative for virus
5	N	N	N	N	N—HeLa C—MK	Virus present that is not poliovirus or is atypical poliovirus
6	C	C	C	C	C	1) Poliovirus but too concentrated for antiserum 2) Poliovirus and other virus 3) Virus other than poliovirus
7	C	C	C	N	C	More than one strain of poliovirus

* N = cells appear normal. C = cells show cytopathic effect of virus.

TABLE II. Concentration of Poliovirus in Supernates and Sediments of Stool Suspensions after High Speed Centrifugation.*

Exp.	Specimen No.	Supernate	Sediment
1	794	68×10^5	99×10^5
	870	33×10^5	38×10^5
	896	112×10^5	57×10^5
2	1364	<1	40×10^5
	1148	274	26×10^6
	144	11×10^2	28×10^5
	1305	2	25×10^5
	855	<1	<2
	837	<1	<2
3	F	40	600
	G	20	500
	H	20	580

* Ten ml of stool suspension was centrifuged and sediment resuspended in 2 ml. Titers expressed as plaque forming units/ml.

these cultures and the test is repeated using MK cell cultures.

The results shown in row 6 indicate that either the amount of virus in stool concentrate is in excess of the neutralizing capacity of the antiserum, or both poliovirus and another virus are present, or a viral agent other than poliovirus is present. The test is then repeated with stool concentrate diluted 10^{-3} and 10^{-5} . Finally, if the result shown in row 7 is obtained, it indicates that 2 types of poliovirus are present. The test is repeated with appropriate pairing of poliovirus antisera (*i. e.*, 1 and 2, 1 and 3, 2 and 3).

Control cultures (f, g, h, i, k) should remain normal in appearance for duration of test, while control (j) should show characteristic cytopathic effect of poliovirus. Any other reactions invalidate part or all of the test.

Studies on efficiency of centrifugation: The essential feature of the present procedure that makes the simultaneous test feasible is the partial purification of virus with concomitant elimination of cytotoxic substances. To ascertain that centrifugation was efficient and that the bulk of virus was not being discarded with the high speed supernate, concentration of virus in the supernate and sediment was measured. The assays were performed by plaque technique and results are contained in Table II. In all cases, except for the last 2 specimens of Exp. 2, which contained no detectable virus, concentration

of virus in sediment was at least 5-fold greater than in supernate. This is true even for specimens 144, F, G, H which contained comparatively little virus. Concentrations of virus in supernates of specimens 1364, 1148 and 1305 are unexpectedly low in comparison with sediments. It seems unlikely that efficiency of centrifugation is the sole explanation, and other possibilities are under study.

Results of studies on stool specimens: Studies on stool specimens from 176 suspected cases of poliomyelitis were carried out using the consecutive (*i. e.*, isolation followed by identification) and simultaneous procedures in parallel. Of 60 specimens found to contain poliovirus by the simultaneous method, 58 were positive by the consecutive method. Although 18 specimens caused non-specific cytotoxic reactions by the latter method, no such reactions were observed with stool specimens prepared by centrifugation.

With the newer technique, 80% of the positive specimens were typed within 5 days of receipt. The remaining specimens were poliovirus strains which either grew slowly or grew only in MK cell cultures.

The above procedure is equally suitable for isolation and identification of other enteroviruses. In several instances, presence of virus was evident in MK cell cultures but not in HeLa cultures, and these isolates were shown to be Echo viruses. Several Coxsackie viruses were also isolated. Three specimens were found to contain dual agents, one contained types 1 and 3 polioviruses, and 2 contained type 1 poliovirus together with a Coxsackie virus.

Summary. A method is described for simultaneous isolation and identification of poliovirus in stool specimens. The principal feature of the method is partial purification of virus by differential centrifugation of stool extract. Virus so prepared is free of non-specific cytotoxins and is also more concentrated than in the original specimen. Results of studies on 176 stool specimens have shown that the method is rapid, sensitive, and free of complicating non-specific reactions. Positive specimens are typed in most cases within 5 days. The method should also enable more

rapid isolation and identification of other enteroviruses.

1. Paul, J. R., Melnick, J. L., in *Diagnostic Procedures for Virus and Rickettsial Diseases* 2nd Edit. Am. Public Health Assn., New York, 1956.

2. Melnick, J. L., *Am. J. Public Health*, 1958, v48, 1170.

3. Baron, S., *PROC. SOC. EXP. BIOL. AND MED.*, 1957, v95, 760.

Received January 4, 1961. P.S.E.B.M., 1961, v106.

Extracellular Proteins of Staphylococci.* (26472)

ALAN W. BERNHEIMER AND LOIS L. SCHWARTZ

Department of Microbiology, New York University School of Medicine, New York

Staphylococci are known to release into the environment in which they grow a number of enzymes and toxins. Knowledge of the origin and function of these extracellular proteins may clarify the manner and degree in which they influence virulence and perhaps communicability. To this end strains of differing pathogenicity were selected with a view to examining the number and distribution of proteins that could be revealed by starch gel electrophoresis, a method capable of giving remarkable resolution as has been demonstrated in studies of proteins of serum (1,2) and other materials (3,4).

Materials and methods. Strains. Strains Mendita and 3895 are examples of classically avirulent coagulase-negative staphylococci of the albus type. Strain Mam-der is regarded as occupying a position intermediate between nonpathogenic and pathogenic staphylococci (5). Strains 4423 and 26925 are examples of *Staphylococcus aureus* phage type 80/81 isolated from the human nasopharynx. Strain 4423 was freely communicable among premature infants in a nursery but was accompanied by an abnormally low lesion rate; strain 26925 was obtained from a healthy person who had no history of recent suppurative lesions. Strains Smith-diffuse and Smith-compact are variants of *Staphylococcus au-*

reus originally isolated from a case of osteomyelitis. The former is virulent for mice intraperitoneally and is lysed by phage 44A; the latter is avirulent for mice intraperitoneally and apparently is not phage-typeable (6).

Production and Concentration of Extracellular Proteins. The medium used was similar in composition to that of Gladstone and van Heyningen (7) but was modified to exclude substances of high molecular weight that might be present in small amounts in some of the constituents. Two liters of doubly concentrated medium, pH 7.1 - 7.2, were placed in a 6 × 18 in pyrex cylinder fitted with a plexiglass cover containing a large central hole and 2 smaller holes eccentrically located. A large cellophane sac (uninflated width 2¾ in) containing 2000 ml distilled water was suspended from the plexiglass cover by bringing the open end of the sac through the central hole and attaching it to a short length of large-diameter glass tubing. The latter was closed with a rubber stopper carrying 0.7 mm inlet and outlet tubes so that the solution inside the sac could be mixed during incubation by bubbling with gas. The 2 smaller holes carried additional inlet and outlet tubes for mixing of solution outside the sac. The assembly was autoclaved for 30 minutes at 15 lb per square inch, allowed to cool, and placed in a 36° incubator for temperature equilibration.

The inoculum, consisting of saline-washed cocci derived from 2 ml of a 16-hour broth culture, was aseptically introduced into the cellophane sac. Incubation was allowed to proceed for 24 hours during which time the

* This investigation was supported in part by research grants from Life Insurance Medical Research Fund and Nat. Inst. of Allergy and Infect. Dis., U. S. Public Health Service. The authors are indebted to Dr. David E. Rogers and Dr. Frederick H. Wentworth for cultures as well as for information regarding their history.

culture inside the sac, and the medium outside as well, were continuously bubbled with a mixture of 20% CO₂ and 80% O₂, cotton-filtered, at a rate of approximately 120 bubbles per minute. Heavy growth occurred inside the sac while none occurred, as a rule, in the external solution. After centrifugation the clear supernate was concentrated 5- to 8-fold by pervaporation, dialyzed against running tap water for 12 to 24 hours, and re-pervaporated to 100 to 200 ml. The solution was dialyzed in the cold against 4 volumes of saturated ammonium sulfate for 2 to 3 days, with intermittent mixing, so that it equilibrated at 80% saturation. The precipitate was recovered by centrifugation, dissolved in 10 ml distilled water, and dialyzed in the cold against 5 l distilled water for 2 to 3 days. The solution was centrifuged, the insoluble sediment discarded, and the supernate lyophilized.

Starch gel electrophoresis. The method used was essentially that described by Smithies(1). As a routine, 10 mg staphylococcal protein preparation were subjected to electrophoresis (borate, pH 8.6) following which the gel was sliced in a horizontal plane and stained with amido black(1).

Distribution of Toxic and Enzymatic Activities. The product obtained from strain 4423 was employed. *Hemolytic activity*, apparently alpha hemolysin, was localized (a) by slicing the gel in the broad plane, as for staining, and bringing the cut surface into contact with rabbit blood agar, and also (b) by titrating against rabbit red cells eluates of slices that had been transversely cut at appropriate intervals along the length of the gel. The amounts of staphylococcal product applied to the gel were 1 and 10 mg respectively. Elution was carried out by slow-freezing at -16° each transverse slice together with 1 ml phosphate-buffered saline containing 0.1% bovine serum albumin, followed by thawing and mechanical compression of the gel sections to express fluid. *Lipase* was detected by placing strips of gel on tributyrin agar(8) and incubating for one hour at 37° followed by removal of the gel strips and examination for clearing. Addition of eluates to egg yolk broth (McGaughey and Chu(9)

resulted in the development of opalescence. The most intense effect occurred in zones corresponding to those of maximal lipase activity suggesting that the opalescence is related to lipolytic activity on triglycerides as proposed by Gillespie and Alder(10). *Desoxyribonuclease* was detected by allowing gel strips to remain in contact with one per cent agar containing 0.2% sodium desoxyribonucleate, 0.03 M borate, pH 8.6, and 0.01 M MgSO₄. After 1 hour at 37°C, the gel strips were removed, the agar flooded with 95% ethanol and examined for differences in opacity. *Hyaluronidase* was estimated in eluates using the method of Tolksdorf *et al.*(11). *Staphylokinase* was detected by placing small volumes of eluates on fibrin plates(12) containing plasminogen, and subsequently observing for areas of fibrin digestion. Control plates from which plasminogen was omitted were used to differentiate direct proteolytic action, if present, from that brought about by staphylokinase. Significant amounts of *proteolytic activity* were absent as judged from failure of fibrin to be digested in plates lacking plasminogen. As no *coagulase* could be detected in 0.1% solutions of unfractionated staphylococcal proteins, it appears that little or no soluble coagulase is formed under the conditions used for cultivation.

Results. Yield and nature of extracellular material. Five cultures of the Smith-diffuse strain were grown and processed as described. Bacterial growth, expressed as OD₆₅₀ varied from 0.37 to 0.68, and total weight of non-dialyzable extracellular product obtained from the supernates varied from 156 to 316 mg. Under the conditions of cultivation used, the Smith-diffuse strain formed non-dialyzable substances averaging 45% of its own weight.

Comparable data for all the strains studied are given in the first 4 columns of Table I. Strain 4423 formed as little as 10% of its weight in non-dialyzable substances while the compact variant of the Smith strain produced 68%. The results suggest that strains of staphylococci vary widely in quantity of extracellular material of high molecular weight that they elaborate during growth.

The non-dialyzable products obtained from

TABLE I. Growth, Yield and Properties of Extracellular Products of Diverse Strains.

Strain	Bacterial growth, OD ₆₆₀	Yield of extracellular non-dialyzable product, mg	Wt of product (mg) × 100		OD ₂₆₀ [*] per 0.5 mg	Folin-Ciocalteu color as tyrosine, μg/mg	Orcinol color as ribose, μg/mg
			Wt of growth (mg)				
			%				
Mendita	.615	99	16	1.36	1.38	47	18.5
3895	.452	190	43	.904	1.26	57	12.4
Mam-der	.462	175	39	1.25	1.55	69	23.6
4423	.660	65	10	1.05	.95	94	7.5
26925	.390	84	22	.83	1.02	69	6.7
Smith-compact	.290	195	68	.85	1.12	61	7.5
Smith-diffuse	.445	186	43	.925	1.07	66	9.3

* 0.03 M borate buffer, pH 8.6.

the culture supernates were assayed for protein using the Folin-Ciocalteu phenol reagent (13) and the results (Table I) indicate that the products consist very largely of protein. However, the values of the ultra-violet absorption ratio, OD₂₆₀ : OD₂₈₀, suggest that substantial amounts of nucleic acid are also present. The products were therefore analyzed for ribose, using the orcinol reagent (14), and from the color developed (Table I) it can be estimated that ribonucleic acid is present to the extent of 3 to 12% of the weight of the products. It is notable that the products of the 2 coagulase-negative strains and the "intermediate" strain contain distinctly larger amounts of ribonucleic acid (9.3 to 11.8%) than those of the 4 coagulase-positive strains (3.8 to 4.7%).

Description and analysis of electrophoretic patterns. The 4 examples of potentially pathogenic staphylococci (strains 4423, 26925, Smith-diffuse and Smith-compact) each show 12 to 14 bands and the patterns of these 4 strains are remarkably similar although not identical. All 4 strains show 3 bands of protein (lettered A, B and C in Fig. 1) which have separated to the left of the sample application slit. (Band B could not be detected in the starch slice of Smith-compact chosen for portrayal in the figure but was present as a faint band in a pattern of another preparation of the same strain). Band B may be of particular interest because it is the heaviest staining band of the 12 formed by the penicillin-resistant phage type 80/81 strain 4423, and because it, as well as bands A and C, is absent from Mendita, 3895

and Mam-der. Labelling of bands of different strains by the same letter does not imply that these bands necessarily represent the same substance but is intended to indicate similarity to the observer's eye, of staining intensity, width, or position in relation to other bands.

The bands labelled H, I, K and L of strain 4423 appear to recur in Smith-diffuse and

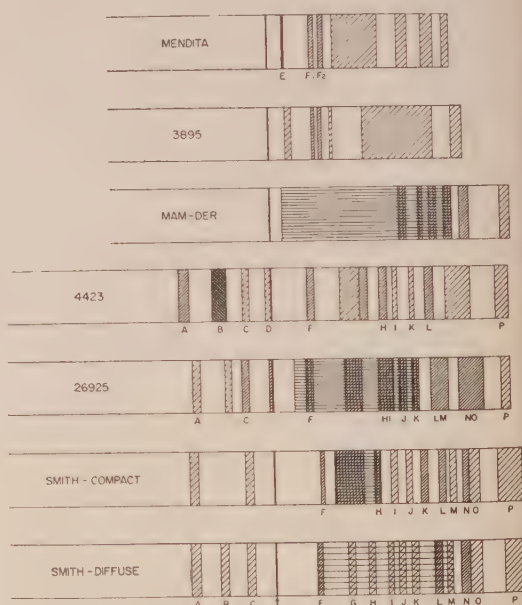


FIG. 1. Representation of starch gel electrophoretic patterns for 7 strains of staphylococci. Arrow shows location at which samples were applied. Bands E, F, G, etc. are in the direction of the positive electrode. Diagonal shading indicates degree of intensity of protein staining; horizontal shading indicates a moderately dark background of diffusely staining material. Because the representation is diagrammatic the sharpness and clarity of the patterns tend to be somewhat exaggerated.

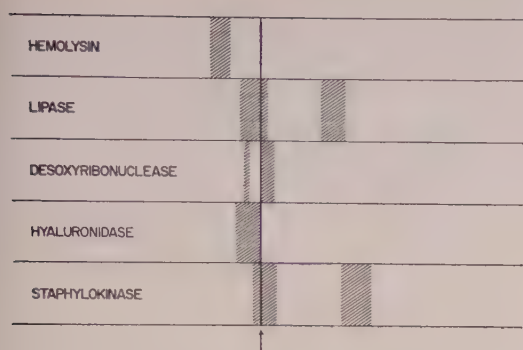


FIG. 2. Localization of hemolytic and enzymatic activities after electrophoresis in starch gel. Strain 4423. Arrow indicates origin. Migration toward the right is in the direction of positive electrode.

Smith-compact. It is less certain that some of the bands of Mam-der are homologous with H, I, K and L of 4423 and the Smith strains, and it is likewise difficult to relate to H, I, J and K the bands in the corresponding region of the 26925 pattern. It is notable that a fast-moving band, P, is present in all patterns except those of Mendita and 3895.

The patterns of the 2 nonpathogenic strains, Mendita and 3895, differ strikingly from those of the potentially pathogenic staphylococci. Only 7 and 6 bands, respectively, are evident and about half of them are relatively faint. The pattern of the "intermediate" strain, Mam-der, shows no very strong resemblance to either those of the pathogenic staphylococci or to those of Mendita and 3895.

To make sure the observed bands actually represent products of staphylococcal growth, medium was assembled, incubated and concentrated in the usual way but instead of inoculating it, 15 mg crystalline ovalbumin were added as a carrier to the medium to be processed. Electrophoresis of the resulting concentrate yielded a pattern indistinguishable from that given by a solution of crystalline ovalbumin, no additional bands being discernible.

The information provided by assays for toxic and enzyme activities is summarized in Fig. 2 in which shaded areas represent zones of maximal activity. In some instances the width of the zones is arbitrary inasmuch as increasing the sensitivity of the test, as can be done by lengthening the time of develop-

ment, tends to broaden the zone of detectable activity. Although lipase and staphylokinase each show 2 zones of maximal activity, it need not be inferred that staphylococci synthesize or secrete 2 kinds of lipase and 2 kinds of staphylokinase. It may be that some of the enzyme molecules are free to move in the gel while the remainder may be aggregated or complexed with a second substance that tends to retain the enzyme at or near the sample slit.

Discussion. The results show that growth of both potentially pathogenic (coagulase-positive) and nonpathogenic (coagulase-negative) staphylococci is accompanied by the accumulation of a large amount of extracellular substance, chiefly protein. Quantities of protein found are comparable with those reported by Rogers(15). The presence of appreciable amounts of ribonucleic acid in the culture supernates here studied indicates that cell lysis occurred to a significant degree, and at first sight (Table I) it appears that lysis occurred to a greater extent among nonpathogenic than among potentially pathogenic strains. Organisms of the latter type, however, are known(16,17) to produce a thermostable nuclease which degrades both DNA and synthetic polyribonucleotides. Ribonuclease activity was readily detectable in the extracellular products of potentially pathogenic strains and it is possible that this enzyme accounts for the difference in orcinol-reactive material found. Even though extensive autolysis occurred in cultures of nonpathogenic staphylococci the electrophoretic patterns yielded by them are distinctly less complex than those of the potentially pathogenic strains.

The existence of a multiplicity of extracellular staphylococcal proteins is deducible not only from the diversity of biological and biochemical effects exhibited by cultures and culture filtrates(18) but also from the results of studies based on an immunological double diffusion technic(19) which revealed as many as 10 antigens to be associated with the Wood 46 strain. Using commercial antitoxin prepared against this strain, staphylococci isolated from lesions produced, on the average, between 6 and 7 lines of precipitate while co-

agulase-negative strains formed none. Further work in this direction(20) showed the number of lines of precipitate to be directly related to mouse virulence.

The technics used in this investigation permit detection of as many as 15 diffusible products (14 stainable bands and one white band) in the case of coagulase-positive staphylococci, and the majority of these, judging from the patterns of activity shown in Fig. 2, do not appear identifiable with previously described staphylococcal enzymes and toxins. It is notable also that many of these proteins are absent from cultures of saprophytic strains. The number of strains that have been examined is small but the results suggest that investigation of the new proteins, and possibly those of additional strains, may yield information that will help elucidate the problem of staphylococcal pathogenicity.

Summary. Strains representative of potentially pathogenic and nonpathogenic staphylococci were studied with respect to the extracellular proteins that accumulate in cultures. Starch gel electrophoresis of the non-dialyzable substances isolated from culture supernates revealed that 12 to 14 species of proteins are elaborated by potentially pathogenic strains while about half as many were found to be associated with nonpathogenic staphylococci. Additional findings permitted the loci of hemolytic and several enzymatic activities to be mapped and compared with those of protein. Most of the proteins do not appear to be identifiable with previously described products of staphylococcal growth, and therefore, represent new extracellular

substances. Several of these occur consistently and perhaps exclusively in association with potentially pathogenic strains.

1. Smithies, O., *Biochem. J.*, 1955, v61, 629.
2. ———, *ibid.*, 1959, v71, 585.
3. Poullick, M. D., *J. Immunol.*, 1959, v82, 502.
4. Markert, C. L., Moller, F., *Proc. Nat. Acad. Sci.*, 1959, v45, 753.
5. Smith, J. M., Dubos, R. J., *J. Exp. Med.*, 1956, v103, 87.
6. Hunt, G. A., Moses, A. J., *Science*, 1958, v128, 1574.
7. Gladstone, G. P., van Heyningen, W. E., *Brit. J. Exp. Path.*, 1957, v38, 123.
8. Marks, J., *J. Path. and Bact.*, 1952, v64, 175.
9. McGaughey, C. A., Chu, H. P., *J. Gen. Microb.*, 1948, v2, 334.
10. Gillespie, W. A., Alder, V. G., *J. Path. and Bact.*, 1952, v64, 187.
11. Toksdorf, S., McCready, M. H., McCullagh, D. R., Schwenk, E., *J. Lab. Clin. Med.*, 1949, v34, 74.
12. Astrup, T., Müllertz, S., *Arch. Biochem. and Biophysics*, 1952, v40, 346.
13. Heidelberger, M., MacPherson, C. F. C., *Science*, 1943, v97, 405 and v98, 63.
14. Mejbaum, W., *Z. Physiol. Chem.*, 1939, v258, 117.
15. Rogers, H. J., *J. Gen. Microb.*, 1954, v10, 209.
16. Cunningham, L., Catlin, B. W., de Garilhe, M. P., *J. Am. Chem. Soc.*, 1956, v78, 4642.
17. Cunningham, L., *Ann. N. Y. Acad. Sci.*, 1959, v81, 788.
18. Elek, S. D., *Staphylococcus pyogenes and its relation to disease*, Edinburgh, 1959.
19. Elek, S. D., Levy, E., *Brit. J. Exp. Path.*, 1950, v31, 358.
20. Howard, J. G., *J. Path. and Bact.*, 1954, v68, 177.

Received January 6, 1961. P.S.E.B.M., 1961, v106.

Effects of Corticoids, Insulin, and Epinephrine on α -Aminoisobutyrate Accumulation in Muscle of Adrenalectomized Rats.* (26473)

J. EICHHORN, M. FEINSTEIN, I. D. K. HALKERSTON AND O. HECHTER

Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Insulin has been shown to increase the accumulation of the "non-utilizable" amino acid, α -aminoisobutyrate (AIB) *in vitro* in diaphragm muscle from intact rats(1) and *in vivo* using functionally nephrectomized, hypophysectomized rats(2). Early in the present work it was noted that the distribution of AIB in muscle of adrenalectomized rats after administration of insulin is about half of that observed in muscles of intact or hypophysectomized rats after insulin. Accordingly, the effects of epinephrine, deoxycorticosterone (DOC), and cortisol administration upon muscle AIB accumulation in adrenalectomized rats with and without the administration of insulin have been studied. The results show that corticoids and epinephrine, independently of each other, enhance the effect of insulin upon accumulation of AIB in muscle of adrenalectomized rats.

Methods. Intact, hypophysectomized (2 day), or adrenalectomized (6 day) rats of Charles River SD strain, weighing 120-190 g, were used. Adrenalectomized rats were maintained with 0.9% saline as drinking water. The effect of insulin administration on AIB transport was determined in diaphragm and gastrocnemius muscle in each of these 3 types of animals. Transport of AIB was also studied in these muscles of adrenalectomized rats in absence and presence of insulin after acute administration of epinephrine, and after pretreatment with DOC or cortisol.

The experimental procedures, described previously(2,3), involved functional nephrectomy under Avertin anesthesia, followed by subcutaneous injection of AIB-1- C^{14} , 1.5-2.5 μ c in 0.3 ml water (Volk, S.A. 2.26 μ c/mg). DOC and cortisol were suspended in Special Formula No. 17874 (Upjohn), and administered subcutaneously to adrenalectomized rats under 2 schedules: (a) "maintenance-type" dosage, 0.8 mg each day for 6 days,

beginning on day of adrenalectomy with none on day of experiment (total, 4.8 mg/rat); (b) short-term dosage in three injections (1.6 mg ea) 6, 4, and 2 hr before sacrifice on the 7th day after adrenalectomy (total, 4.8 mg/rat). 1-epinephrine bitartrate (100 μ g/rat, equivalent to 55 μ g epinephrine) was injected subcutaneously to adrenalectomized rats immediately after functional nephrectomy at a site distant from the subsequent AIB injection. Each group of rats (intact, hypophysectomized, adrenalectomized, and adrenalectomized treated with either epinephrine or corticoids) was divided so that part received insulin (1U/100 g) intraperitoneally accompanied by 100 mg glucose/100 g to prevent hypoglycemia; the remainder of each group received 25 mg glucose/100 g as a control injection.

Rats were reanesthetized (Nembutal) 90-100 min after AIB injection (which was also 90-100 min after epinephrine or insulin administration); plasma was obtained immediately from aortic blood, and tissues (diaphragm and gastrocnemius muscle) were excised without delay. Tissues were extracted with water at or near 100° for 15 min. The diluted (1:50) plasma and tissue extracts were plated and counted in a gas flow chamber. A previous report demonstrates that the radioactivity measured in this manner represents primarily unchanged AIB(2). Dosage of AIB used gave a concentration in plasma of 0.1 to 10 μ g/ml, which is within the range previously found to yield distribution values in diaphragm which are relatively independent of alterations in plasma concentration (2).

Results. In Table I, the percentage distribution of AIB between tissues and plasma is expressed as the ratio, 100 \times cpm per g wet tissue/cpm per ml plasma. AIB distributions in both muscles of untreated intact rats are significantly higher ($p = <.001$) than those in hypophysectomized or adrenal-

* This work is supported by U.S.P.H.S., Nat. Science Foundation, and the Commonwealth Fund.

TABLE I. Percent Distribution of AIB between Muscles and Plasma of Intact, Hypophysectomized, or Adrenalectomized Rats, and Influence of Various Hormones.

	Hormone treatment*		AIB: $\frac{\text{cpm/g wet tissue}}{\text{cpm/ml plasma}} \times 100$	
	Insulin	Other	Diaphragm	Gastrocnemius
Intact	—		157 \pm 12 (18) [†]	93 \pm 6 (4) [†]
	+		385 \pm 44 (6)	121 \pm 13 (7)
Hypophysectomized (2 day)	—		72 \pm 7 (5)	41 \pm 3 (5)
	+		420 \pm 110 (16)	102 \pm 13 (5)
Adrenalectomized (6 day)	—		91 \pm 13 (12)	38 \pm 5 (12)
	+		194 \pm 6 (19)	51 \pm 3 (19)
	—	Epinephrine	87 \pm 9 (8)	55 \pm 4 (8)
	+	"	354 \pm 36 (9)	99 \pm 12 (10)
	—	Cortisol (1-6 day)	154 \pm 23 (10)	59 \pm 8 (9)
	+	"	280 \pm 30 (13)	75 \pm 8 (13)
	—	DOC (1-6 day)	144 \pm 12 (3)	72 \pm 5 (3)
	+	"	497 \pm 62 (5)	144 \pm 16 (5)
	—	Cortisol (7th day)	67 \pm 4 (10)	34 \pm 3 (10)
	+	"	147 \pm 16 (11)	50 \pm 5 (12)
	—	DOC (7th day)	89 \pm 10 (6)	39 \pm 4 (6)
	+	"	192 \pm 28 (6)	43 \pm 5 (6)

* Dosage of hormone: Insulin, 1 U/100 g body wt; glucose with insulin, 100 mg/100 g; control glucose, 25 mg/100 g; epinephrine, 55 γ /rat; DOC and cortisol, total of 4.8 mg/rat in divided doses as described in text.

[†] Mean, stand. error, and No. of observations.

ectomized rats; no significant difference exists between the latter two groups. Administration of insulin increases AIB distribution about 2.5 times in diaphragm of intact rats, and 5.8 times in diaphragm of hypophysectomized rats; the respective increases in gastrocnemius are 1.3 times and 2.5 times the control level. In adrenalectomized rats, AIB distribution after insulin administration is increased 2.1 times in diaphragm, and 1.3 times in gastrocnemius. While the percentage increase in AIB distribution produced by insulin administration in muscle of adrenalectomized rats is approximately equivalent to the response in intact rats, the absolute level of AIB distribution achieved in adrenalectomized rats is about half that in muscle of intact rats. In hypophysectomized rats, where the level of AIB distribution in absence of exogenous insulin is statistically equivalent to that in untreated adrenalectomized rats, insulin increases AIB distribution in both muscles to values comparable to those in intact rats. Exogenous epinephrine alone in the dosage used has no effect in diaphragm, but slightly increases AIB distribution in gastrocnemius ($p = <.02$) of the adrenalectomized

rats. However, when epinephrine is given in addition to insulin, the absolute levels of AIB distribution in both muscles are increased significantly above those obtained with insulin alone ($p = <.001$), giving values which are not significantly different from those after insulin treatment in intact or hypophysectomized rats.

"Maintenance-type" dosage with either cortisol or DOC in absence of exogenous insulin results in AIB distribution values in diaphragm comparable to those in untreated intact rats; these corticoids significantly increase the AIB distribution values in gastrocnemius above the untreated adrenalectomized level, but not to that in the untreated intact rat. After insulin administration to adrenalectomized rats treated with either DOC or cortisol for 6 days, AIB distributions in diaphragm are increased to a level which is statistically indistinguishable from values obtained in diaphragm of intact rats treated with insulin. However, the absolute level of AIB distributions with DOC plus insulin is significantly greater in diaphragm than with identical doses of cortisol plus insulin ($p = <.01$). In gastrocnemius, treatment with

either corticoid increases the level of AIB distribution over the non-treated adrenalectomized control; as in diaphragm, insulin administration increases the absolute AIB distribution values to a significantly greater extent in DOC-treated rats than in cortisol-treated rats. When given on the "short-term" basis, beginning 6 hr before sacrifice, neither corticoid elevated the distribution of AIB in either muscle in absence or presence of exogenous insulin.

Discussion. The present study demonstrates that, while insulin administration produces an increase in AIB distribution in muscle of adrenalectomized rats which on a percentage basis is equivalent to the insulin-induced increase in muscle of intact rats, the absolute level of muscle AIB distribution after insulin is much reduced in adrenalectomized rats relative to intact or hypophysectomized rats. The contrast between the effect of insulin in adrenalectomized and hypophysectomized rats is especially striking, since the values obtained in muscles of untreated rats in these 2 groups are about equivalent. Thus, in hypophysectomized rats, the response to insulin in muscle is markedly increased over adrenalectomized rats both on the basis of percentage change after insulin and in terms of absolute level of AIB distribution achieved. It thus appears that presence of the adrenal is required for the maximal effect of a given dosage of insulin on AIB accumulation in muscle.[†] Either epinephrine or maintenance-dosage with corticoids, independently of each other, clearly enhances the AIB accumulation effect of insulin in muscle, and it appears that both the adrenal cortex and medulla are implicated in the lower AIB distribution observed after insulin administration to adrenalectomized rats.

Since exogenous epinephrine augments the action of injected insulin to increase muscle AIB accumulation in adrenalectomized rats, it is possible that the elevated response to injected insulin observed in muscle of the in-

tact and hypophysectomized rats relative to adrenalectomized rats is dependent in part upon the release of endogenous epinephrine induced by operative stress and/or insulin administration. Noall *et al.* (6) report a tendency for epinephrine to increase AIB accumulation in "muscle" of adrenalectomized rats, but the effect was not statistically significant. Because it might be expected that injected epinephrine would stimulate release of endogenous insulin, the question arises as to why epinephrine injected alone did not produce an "insulin effect" in muscle of adrenalectomized rats. This may be due to the fact that the hyperglycemic response to epinephrine, important for endogenous insulin release, is much reduced in rats following adrenalectomy (4,5).

In regard to the role of corticoids, it appears that long-term treatment of adrenalectomized rats with a glucocorticoid, cortisol, is less effective than is treatment with a mineralocorticoid, DOC, in restoring the ability of muscle to respond to insulin in terms of AIB accumulation against an apparent gradient. These effects of corticoids on AIB transport appear to be indirect, since short-term corticoid treatment did not enhance AIB uptake in adrenalectomized rats, with or without exogenous insulin. Since DOC, and to a lesser extent, cortisol, affect electrolyte metabolism, the findings suggest that maintenance of a suitable ionic balance between cells and plasma may have an important influence upon the process by which AIB is accumulated in muscle, and that the lower accumulation of AIB observed in the adrenalectomized rat after insulin administration may be related to the high plasma K^+ level characteristic of this type animal. This suggestion is in accord with *in vitro* studies, which indicate that high K^+ levels in the medium inhibit glycine transport into isolated diaphragm, and also that ionic shifts are associated with active transport of several amino acids (*cf.* 7). In the present study, plasma $[K^+]$ in adrenalectomized rats ($5.85 \pm 0.36 \mu\text{M/ml}$ $n = 18$) is higher than in intact rats ($3.74 \pm 0.05 \mu\text{M/ml}$ $n = 4$), or in rats hypophysectomized for 2 days (3.68 ± 0.15 , $n = 4$). Administration of

[†] In regard to results in hypophysectomized rats, it cannot be assumed that residual actions of pituitary hormones, particularly somatotrophin, were entirely absent in 2-day hypophysectomized rats.

epinephrine to adrenalectomized rats, shown to augment muscle AIB accumulation after insulin, significantly reduces the elevated plasma $[K^+]$ of adrenalectomized rats to 4.50 ± 0.28 ($n = 4$), but 6 hr pretreatment with either DOC or cortisol, which had no effect on muscle AIB response to insulin, did not reduce plasma K concentration. Values for other treatments were not obtained, but these preliminary observations indicate that the relationship of plasma $[K^+]$ to the insulin-induced increase in muscle AIB accumulation merits further study.

Independent of the foregoing, the results of this study suggest that epinephrine and corticoids may play a "permissive" or "supportive" role, in the sense proposed by Ingle (8), in the response of muscle to insulin with respect to AIB transport.

Summary. The distribution of AIB *in vivo* in diaphragm and gastrocnemius muscle of intact, hypophysectomized, and adrenalectomized rats has been examined with respect to the effect of insulin and epinephrine and corticoids. Adrenalectomy, but not hypophysectomy, impairs the ability of insulin to increase the accumulation of AIB in muscle to the level observed after insulin in intact rats. Epinephrine alone has slight effect on muscle AIB in adrenalectomized rats, but

concurrent administration of epinephrine and insulin to adrenalectomized rats restores AIB distribution nearly to the level observed in adrenal-intact rats receiving insulin. Maintenance treatment of adrenalectomized rats with either DOC or cortisol resulted in normal AIB distributions in diaphragm and both corticoids, especially DOC, also enhance the AIB accumulation response to insulin. The suggestion emerges that these hormonal effects on AIB accumulation may be related to alterations in electrolyte metabolism.

1. Kipnis, D. M., Noall, M. W., *Biochim. et Biophys. Acta*, 1958, v28, 226.
2. Eichhorn, J., Scully, E., Halkerston, I. D. K., Hechter, O., *Proc. Soc. Exp. Biol. and Med.*, in press.
3. Eichhorn, J., Halkerston, I. D. K., Feinstein, M., Hechter, O., *ibid.*, 1960, v103, 515.
4. Collip, J. B., Thomson, D. L., Toby, G., *J. Physiol.*, 1936, v88, 191.
5. de Bobo, R. C., Bloch, H. I., Gross, I. H., *Am. J. Physiol.*, 1942, v137, 124.
6. Noall, M. W., Riggs, T. R., Walker, L. M., Christensen, H. N., *Science*, 1957, v126, 1002.
7. Christensen, H. N. *Amino Acid Metabolism*, Ed. W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, 1955, p63.
8. Ingle, D. J., *J. Endocrinol.*, 1952, v8, xxxiii.

Received January 10, 1961. P.S.E.B.M., 1961, v106.

Effect of External Cooling of the Leg on Muscle Intracellular Water and Potassium.* (26474)

R. KLEIN AND PATRICIA USHER

Children's Hospital of Pittsburgh and Department of Pediatrics, University of Pittsburgh Medical School

Patients with the hyperkalemic form of familial periodic paralysis become paralyzed upon exposure to a cold environment(1,2). Localized paralysis of the arm has been produced in experiments with these same patients by swathing the forearm in ice packs for $\frac{1}{2}$ hour(3,4). We have postulated that in this disease there is an exaggeration of the normal flux of potassium from the intracellu-

lar into the extracellular fluid and an inhibition of the normal return of potassium into the intracellular fluid. Muscle biopsies from one of these patients demonstrated an elevated intracellular water concentration and a depression of intracellular potassium concentration, but no direct evidence was obtained that potassium moved out of the muscle when paralysis was produced by external application of cold. The present studies were carried out to test the hypothesis that intra-

* This work was supported by Grant from Nat. Inst. Health, U.S.P.H.S.

TABLE I. Water and Na, K, Cl Content of Leg Muscles after External Cooling of Leg.

	H ₂ O _(i) , ml	Na	K	Cl	Na	K
		meq			meq/l (H ₂ O) _i	
	100 g of dry fat-free solids					
Control (38 rats)	276.0 ± 2.72*	9.5 ± .29	48.0 ± .31	5.0 ± .11	9.4 ± .86	173.4 ± 1.85
Exp. (30 rats)	284.6 ± 2.38	10.1 ± .26	46.7 ± .36	5.4 ± .16	9.4 ± .66	164.3 ± 2.19
Significance of difference	p < .01	p > .1	p < .01	p < .05	p > .1	p < .01

* ± stand. error of mean.

cellular potassium concentration diminishes in the muscle of a locally cooled leg of the intact animal.

Methods. White, male rats were anesthetized with intraperitoneal pentobarbital, and the right hind leg was held immersed in a Petri dish containing acetone and ice for 30 minutes. Control rats were similarly anesthetized and immobilized, but the leg was immersed in a Petri dish containing only tepid water. The tests were carried out on groups of 6 to 8 control, and 6 treated animals. After 30 minutes of cold immersion, the middle portion of the rectus femoris muscle was removed, dried on filter paper and placed in tared crucibles for weighing. After the wet weights had been obtained, the muscles were dried in an oven at 105°C until constant dry weights were obtained. The dry muscle was pulverized. The fat of the dried muscle was then extracted with petroleum and ethyl ether. The ether was discarded and the muscle residue once more dried in the oven at 105°C. The muscle was reweighed to obtain the weight of the fat removed and the residue then dissolved in 2 ml of 0.75 N nitric acid. From this, aliquots were taken for sodium, potassium and chloride analysis. Sodium and potassium determinations were performed using a flame photometer with an internal lithium standard. Chloride determinations were made with the Cotlove Chloridometer. Muscles from control animals were treated in the same fashion. Muscles were also removed for analysis from the left hind limbs of 19 of the animals whose right hind limb was chilled. No exact measurements of muscle temperature were made. At the end of experiment the temperature of the cold bath with the immersed right hind limb was 0-3°C. When exposed, the cold

muscles were of normal color and appearance and of normal contractility when cut.

Chloride in the tissue was used as an indicator of extracellular water in our computations. The hazards of this calculation are recognized. However, any increase in intracellular chloride in the cooled muscles would accentuate the differences.

Results. The data recorded in Table I show that the muscles from the 38 control animals contained an average of 276.0 ± 2.72 ml (SEM) of intracellular water per 100 g of dry fat free solid. Mean intracellular water content of muscles from the chilled legs was 284.6 ± 2.38 ml (SEM) per 100 g of dry fat free solid. This difference was significant at the 99% level of confidence. Mean potassium concentration in control muscles was 173.4 ± 1.85 meq/l of intracellular water, whereas in the 30 muscles from limbs exposed to cold, mean potassium concentration per liter of intracellular water was 164.3 ± 2.19 meq. This difference and that between potassium concentrations per 100 g of dry fat free solids were also significant at the 99% level of confidence. The sodium per 100 g of dry fat free solids was insignificantly lower in control muscles than in muscles exposed to cold. Concentration of sodium in intracellular water in the 2 groups of muscles did not differ. Intracellular water concentration and concentration of potassium per liter of intracellular water in the muscles obtained from the limb opposite the cooled extremity in the experimental animals were measured only for the first 19 animals since it was impracticable to prevent some chilling of the left hind limb as well as the right hind limb and it was impossible to determine the extent of this chilling. In these 19 muscles, mean intracellular water concentration was

283 ml/100 g of dry fat free solids. There were 170 meq/k per liter of intracellular water.

Discussion. Interpretation of these findings is complicated by possible variations in blood flow in the cooled hind leg and the stressful nature of the procedure. However, the conditions employed for the experiment reproduced as closely as possible those used in production of localized paralysis in patients with hyperkalemic familial periodic paralysis. In the latter experiments, the forearm was swathed with ice bags for 30 minutes and paralysis was produced(3,4). In these experiments with rats, the changes in water and potassium concentration of the muscles demonstrated are analogous to those previously posulated for the patient with hyperkalemic familial periodic paralysis. Thus, water enters the muscle cell, significantly increasing water content in relation to dry solids of muscle. Presumably, sodium accompanies the water since there is no change in sodium concentration per liter of intracellular water. Concentration of potassium per liter of intracellular water in muscles of the limb exposed to cold becomes significantly decreased. No measurements have been made of muscle water and potassium in patients with hyperkalemic familial periodic paralysis after exposure to cold. Under other circumstances, however, muscle specimens obtained at biopsy from these patients have been shown to contain an increased quantity of intracellular water and a depression of potassium concentration per liter of intracellular water(3,4).

Published reports of intracellular potassium changes during hypothermia have been conflicting. Spurr, Swan, and Taylor found no decrease in cardiac muscle potassium in the hypothermic animal and at times demonstrated actual increases in potassium concentration(5,6,7) but Taylor found decreases in skeletal muscle potassium although Renkin did not(7,8). On the other hand, Gollan reported decreases in concentration of potassium in both cardiac and skeletal muscles in hypothermic animals(9). Finally, Beavers has reported an increase of potassium concentration in cardiac muscles, but a decrease

in skeletal muscle in animals undergoing hypothermia(10). He demonstrated an increased intracellular water concentration in both cardiac and skeletal muscles in these animals. All observers have agreed that plasma concentration of potassium is lowered during hypothermia (as it was with local hypothermia in patients with hyperkalemic familial periodic paralysis). Many of the differences have resulted from *in vitro* as opposed to *in vivo* experiments or immersion as opposed to perfusion chilling, etc. Our data on potassium and water content of skeletal muscle from a chilled leg of a rat show the same deviation from control values that Beavers found in animals made totally hypothermic. It cannot be stated whether the smaller changes seen in muscles of the opposite leg were produced by local hypothermia of this limb as well or by cooling elsewhere. It is also impossible to state whether the changes were a direct effect of cooling or were secondary to diminished blood supply and possible anoxia. Nevertheless, the results of this study are consistent with the hypothesis that local exposure of a leg to cold is associated with an increase in that limb in muscle intracellular water and a decrease in muscle potassium concentration without measurable change in sodium concentration. These findings are not inconsistent with the previous postulations concerning patients with hyperkalemic familial periodic paralysis. If these postulations are correct, it would be expected that the changes in such patients on exposure to cold would be of greater magnitude than those demonstrated in normal rats.

Since intracellular content of potassium in muscle decreases at the same time that extracellular potassium concentration falls (without demonstrable increase in extracellular volume) the potassium content of another tissue or compartment must increase with hypothermia. The two obvious areas to be considered first are the viscera, particularly the liver, and the bones. Although this question must still be investigated further, we think that the bones are not the most likely site of potassium gain, because in the previously described human experiments the measurements of A-V differences in the forearm

should have involved bones as well as soft tissues(4). Unfortunately, these data are capable of other interpretations. Attempts to demonstrate increased potassium content in rat liver have not been successful.

Summary. Measurements were made of muscle intracellular water, potassium and sodium concentrations in 2 groups of rats. The right hind limb of the experimental animals was immersed in a bath of acetone and ice, the right hind limb of the control animals in a bath of tepid water. Exposure of the leg to external cooling was associated with an increase in intracellular water in the muscles of that leg and a decrease in potassium concentration per liter of intracellular water. There was no difference in sodium concentration in the muscles from the chilled extremity from that of the control muscles.

1. Gamstorp, I., Hauge, M., Helivey-Larsen, H. G., Mjones, H., Sagild, U., *Am. J. Med.*, 1957, v23, 285.
2. Egan, T., Klein, R., *Pediatrics*, 1959, v23, 76.
3. Klein, R., *Maandschrift Voor Kindergeneeskunde* 1960, v28, 60.
4. Klein, R., Egan, T., Usher, Patricia, *Metabolism*, 1960, v9, 1005.
5. Spurr, G. B., Barlow, G., *Circulation Research*, 1959, v7, 210.
6. Swan, H., Nat. Res. Council, Nat. Acad. Sci., *The Physiology of Induced Hypothermia*, 1956, v451, 42.
7. Taylor, I. M., *ibid.*, 1956, v451, 26.
8. Renkin, E. M., *ibid.*, 1956, v451, 32.
9. Gollan, F., Rudolph, G. G., Olsen, N. S., *Am. J. Physiol.*, 1957, v189, 277.
10. Beavers, R. W., Rogers, J. J., Jr., *Am. J. Physiol.*, 1959, v196, 706.

Received January 13, 1961. P.S.E.B.M., 1961, v106.

Chemotherapeutic Studies on Cutaneous Leishmaniosis. (26475)

N. ERCOLI (With the Assistance of Rose Marie Fink)

Dept. Experimental Therapeutics, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela

The majority of experimental studies on antileishmanial drugs were conducted on visceral leishmaniosis. The method most frequently used for testing chemotherapeutic agents is that recommended by van Dyke and Gellhorn(1) using hamsters infected intraperitoneally with *L. donovani*. Stauber *et al.*(2,3) recently described a method which permits rapid completion of the test by counting the number of organisms in liver impression smears 8 days after a heavy intracardial inoculation with *L. donovani*.

Being interested in the therapy of the cutaneous forms we utilized an infection to which the mouse is quite susceptible, obtained with a strain of *L. brasiliensis* from a clinical condition—leishmaniosis tegumentaria diffusa—described by Convit(4). In man, this infection attacks the entire skin surface and resembles lepromatous leprosy. The disease does not affect the viscera and mucous membranes, though it sometimes induces a slight infiltration around the lower

nasal septum. The cutaneous lesion is formed "by a specific granuloma composed of large macrophages, which are practically full of parasites in their highly vacuolated protoplasm"(5). Mayer, Convit and Pifano(6) have experimentally investigated the infection and induced lesions on the caudal zone of the back in mice, first in ulcerative, later in nodular form, with an impressive abundance of parasites. Pifano(7) in a geographical distribution study concludes that the American tegumentary leishmaniosis is formed by various sub-entities (espundia, uta, *úlceras de los chicleros*), one of them being represented by the leproid form considered here. Interestingly enough, the intradermal Montenegro test with phenolated suspensions of *L. brasiliensis*(8) remained negative only in the latter clinical manifestation (5,6).

The possibility to follow accurately the effect of the treatment by gross and microscopic observation of the mouse infected with

this strain, gives to each individual experiment the value of a case history. We present here our findings concerning the action of drugs which have been found effective in *L. donovani* infections, namely tri- and pentavalent antimonials, Hydroxy-stilbamidine, the antibiotics Fungizone and Fumagillin.*

Materials and methods. A strain isolated from a chronic clinical case, carried through mice for a number of years by Prof. Pifano, has been used for investigation. In the early part of our experiments, ca. 0.1 cc of exudate taken from a heavily infected lesion was injected with a Pasteur pipette subcutaneously into the caudal zone of mice; later, finely cut saline suspensions of nodules were injected with syringe. In this way, up to 60 mice could be infected with a nodule suspension, dense in parasites, obtained from one donor. The take of the infection varied from 51 to 100% in different experiments. The infection developed after 1 to 3 months in 52%, 3 to 5 months in 32%, 6 to 9 months in 13% and 10 months or more in 3% of 159 mice. In 3 mice the infection was first visible 320 to 350 days after inoculation.

For treatment, animals with well developed lesions, rich in parasites, were selected: they were observed macroscopically and by preparing stained smears of the material aspired from the lesion.

Results. *Tartar emetic* in daily subcutaneous doses of 15 mg/kg, given 52 to 82 times, induced no changes in 5 mice. Similarly, 2 \times 50 mg/kg i.p. remained ineffective in a mouse which died after 10 days. *Antimony trisulfide*, given daily in 150 and 500 mg/kg oral doses induced after the first treatments morphological variations of the leishmania, which appeared larger and granulated in both cases. Besides, significant transitory decrease of the parasites was encountered in the mouse treated with the 150 mg/kg dose; however, during the treatment period the parasite number increased and stayed at pretreatment value after 33 administrations. *Fuadin* induced in

2 mice, after the second subcutaneous treatment with 50 mg/kg, a temporary reduction in number of leishmania with morphological changes consisting in disappearance of nucleus and/or blepharoplast and presence of cytoplasmatic granules in preparations stained with Wright-Giemsa. Yet, by continuing treatment, with 100 mg/kg daily, the organisms reacquired their normal shape and the lesion was larger than initially after the 32nd treatment. *Hydroxy-stilbamidine* was given 14 times in daily doses of 12.5 mg/kg (i.p.) to 4 mice. In 3 animals no effect resulted (in fact, in 2 of them the lesions were larger after treatment), while in 1 out of 4 the lesion decreased and no parasites were encountered after 4 months. *Fungizone*, 20 mg/kg daily (i.p.), given for 14 days, showed no activity in 4 mice, nor did 2 mice injected intravenously with 17 mg/kg \times 3 respond to treatment. *Fumagillin*, injected intraperitoneally 14 times, in daily doses of 300 mg/kg, induced no effect in 3 out of 4 animals. In a fourth, the caudal lesion disappeared after 5 months, to reappear 8 weeks later. *Sodium-stibogluconate* (*Pentostam*), given intraperitoneally in 19 daily doses of 75 mg/kg did not influence the lesions of 3 mice, which became even more extensive during this treatment period. After an additional treatment course of 5 \times 100 mg/kg the nodules started to decrease, but fully redeveloped within 3 months. *Antimoniate of N-methyl-glucamine* (*Glucantime*) in 4 mice treated subcutaneously, twice daily with 500 mg/kg, a total of 52 times, reduced the diameter of the nodules from 0.3-0.6 cm to 0.1 cm. In 2 of the mice the nodules increased again 3 months after cessation of treatment, and a secondary lesion of the foreleg developed in one of them. Four mice were treated 13 times with 1000 mg/kg daily: the larger ulcerated lesions of 2 mice remained unchanged, while in 2, with similar nodules, the lesion continued to increase (from 0.1 to 0.6 cm diameter). Six treatments with 1000 mg/kg daily showed no effect in 3 out of 4 mice, while the lesion disappeared temporarily in one and relapsed after 3 months. One of 2 mice treated 22 times with 500 mg/kg daily did not respond to treatment; in the other

* We are indebted to Drs. R. K. Richards and R. B. Hasbrouck of Abbott Laboratories for Fumagillin, W. L. Koerber and J. N. Pagano of Squibb for Fungizone and to R. S. F. Hennessey, Wellcome Research Laboratories, London, for Pentostam.

the initial lesion was not visible 2 weeks after the last subcutaneous injection, but a secondary lesion rich in leishmaniae appeared in the right hind leg 10 weeks later. In mouse No. 52, treated twice with 1000 mg/kg, the parasite count lowered within 6 days and the lesion disappeared within 4 weeks, but a secondary lesion in the left foreleg appeared 2 months later. The only animal completely cured was another treated with the same dosage: the initial lesion, containing 1000 parasites/field in the exudate smear (400 \times), disappeared completely 10 days after the second injection.

Conclusions. It would appear from the experimental findings that Fungizone, Fumagillin and Hydroxy-stilbamidine, effective against *L. donovani*, possess no action against the strain of *L. brasiliensis* obtained from a patient affected with leishmaniosis tegumentaria diffusa, employed for the cutaneous infection of mice. *In vitro*, Fungizone showed a high leishmanicidal action (0.01 μ g/cc) on a strain of *L. brasiliensis* studied by Furtado *et al.*(9). Prolonged treatment with pentavalent antimonials, Pentostam and Glucantime, induced temporary decrease of the parasite number in the exudate, occasionally a transitory regression of the lesion, but definitely no curative effect. The lesion in most cases reappeared on the initial (caudal) site of the inoculation and exceptionally in another zone, in the legs. The temporary action observed was very clear cut and proves the value of the method for a search of drugs acting more specifically on this infection, or possessing, in a more general sense, a different strain specificity than the known agents. In some cases morphological variations were noted in the early period of treatment (Fig. 1). The significance of these changes is under investigation. There is a parallelism between clinical and experimental results, since according to the experience of the clinicians antimonials give only a temporary regression or diminution of symptoms. In conclusion, one can state that the effect of the presently known antimonials with antileishmanial action in this particular form of cutaneous infection is limited to a temporary effect on the

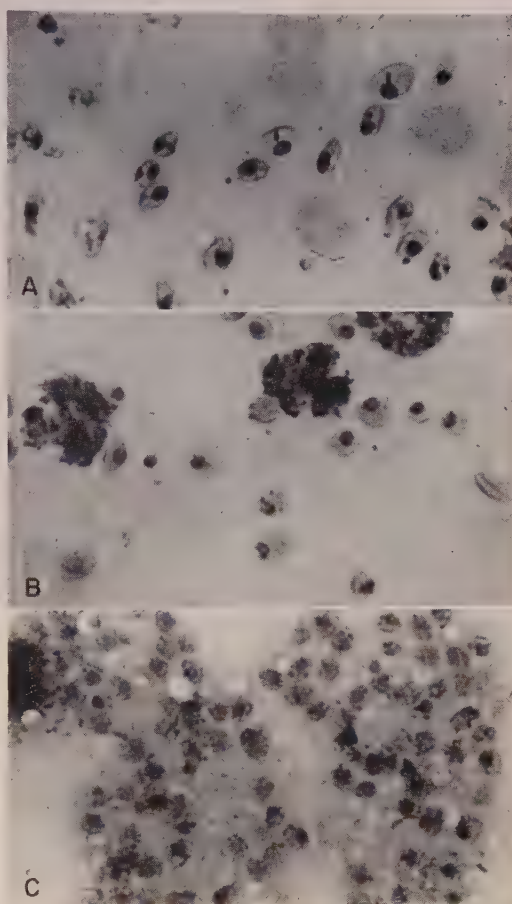


FIG. 1. Smears from local lesions of mice. In *A*, normal appearance of leishmania. In *B*, disappearance of blepharoplast and in *C*, cytoplasmic granules after 2 treatments with 50 mg/kg (s.c.) Fuadin. In both cases these changes were temporary; the parasite reacquired its usual form during successive treatment with an increased dosage (100 mg/kg \times 29).

lesion, or a transitory clearing without curative action.

Summary. The cutaneous infection of mouse obtained with a strain of *L. brasiliensis* causing in man the leproid form represents a suitable test for chemotherapeutic investigation. The infection has not been influenced by the antibiotics Fungizone and Fumagillin recently reported to possess antileishmanial action against *L. donovani*(10). A limited effect followed administration of pentavalent antimonials, consisting in a drop of the parasite number in the exudate and a temporary regression of the lesion, without indication

for curative action. Thus, the drug sensitivity of the strain is totally different from the ones studied so far, which offers the possibility to reveal agents with a different strain specificity than the known ones.

1. van Dyke, H. B., Gellhorn, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, v61, 403.
2. Franchino, E. M., Grun, J., Stauber, L. A., *J. Parasitol.*, 1956, v42, (Sect. 4), 11.
3. Stauber, L. A., Franchino, E. M., Grun, J. J. *Protozool.*, 1958, v5, 269.
4. Convit, J., Lapenta, P., *Rev. Policlínica Caracas* 1948, XVIII, 153.

5. Convit, J., *Rev. de Sanidad y Asistencia Social*, Caracas, 1958, v23, 1.
6. Mayer, M., Convit, J., Pifano, F. C., *Arch. Venezolanas de Patol. Trop. y Parasitol.*, 1949, v1, 183.
7. Pifano, F. C., *Gaceta Méd. de Caracas*, 1960, v68, 89.
8. Mayer, M., Pifano, F. C., *Rev. de Sanidad y Asistencia Social*, Caracas, 1941, v6, 281.
9. Furtado, T. A., Cisalpino, E. O., Santos, U. M., *Antibiot. & Chem.*, 1960, v10, 692.
10. Franchino Cappuccino, E., Stauber, L. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v101, 742.

Received January 17, 1961. P.S.E.B.M., 1961, v106.

Deletion of Serine Dehydrase and Cystathionine Synthetase Activities During Azo-Dye Carcinogenesis. (26476)

DONALD E. KIZER AND DANE EUGENE LACEY* (Introduced by Thomas A. McCoy)

Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Okla.

The Novikoff hepatoma, a tumor induced in rat liver using 4-dimethylaminazobenzene (DAB)(1), was reported to be essentially devoid of serine dehydrase activity(2). Recently, Selim and Greenberg(3) reported that a purified protein from rat liver exhibited both serine dehydrase and cystathionine synthetase activities. The authors suggested that their data indicated both activities were exhibited by a single enzyme(3). Assuming this suggestion to be correct, and further assuming that the enzymatic constitution of transplanted lesions of hepatic origin represented multiple deletions during hepatocarcinogenesis(4,5), it was reasonable to expect that cystathionine synthetase would also be essentially absent in the Novikoff hepatoma. It was the purpose of this investigation to determine (a) whether both serine dehydrase and cystathionine synthetase activities were absent in several transplanted hepatomas and (b) whether both enzyme activities were altered during hepatocarcinogenesis with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB).

Materials and methods. The manner in

which transplanted hepatomas were carried, precancerous liver tissues were obtained, and primary lesions were induced with 3'-Me-DAB have been previously described(6-8). Pyruvic acid formation, determined by the method of Friedemann and Haugen(9), was used as the criterion for assaying serine dehydrase activity of tissue homogenates, while serine utilization, determined by the periodate oxidation method of Frisell *et al.*(10), was used to assay cystathionine synthetase activity. The chromogen formed from chromotropic acid and formaldehyde was measured at 570 m μ and compared to an appropriate standard curve constructed with L-serine. All optical density measurements were made using a Bausch and Lomb Spectronic 20 Colorimeter.

Direct estimation of cystathionine was achieved by one dimensional paper chromatography(11) using n-butanol, acetic acid and water (60:15:25) as the solvent system. Known compounds and supernatants obtained by centrifugation of reaction mixtures treated with 10% trichloroacetic acid (TCA) were spotted on 47 cm \times 57 cm sheets of Whatman No. 3 paper and subjected to descending solvent flow. Following partition,

* Present address: Civil Aeromedical Research Inst., Biochemistry Dept., Norman Okla.

color was developed using 0.25% ninhydrin in acetone(12).

Livers from 3 animals were finely minced with scissors and washed with 1.2% potassium chloride (KCl) containing 5×10^{-3} M ethylenediaminetetraacetate (EDTA). Sufficient tissue (wet weight) was added to KCl-EDTA solutions to yield a 40% homogenate. Tumor tissues were freed of gross necrotic areas and connective tissue elements and were homogenized in a similar manner. The Novikoff ascites cells were occasionally exposed to 15 second sonic oscillation to ensure complete cell disruption.

Cystathionine synthetase activity was determined by adding 1 ml of the 40% homogenate to 3 ml of a reaction mixture containing 0.04 M DL-homocysteine[†], 0.033 M L-serine, 3.2×10^{-3} M EDTA, 5×10^{-5} M pyridoxal phosphate and 1.3×10^{-3} M 2,3-dimercaptopropanol. The total volume of the incubation mixture was brought to 6 ml by addition of 2 ml of 0.2 M borate buffer, pH 8.5. Reaction mixtures were incubated 1 hour at 38°C under 95% nitrogen 5% CO₂. Synthetase activity was determined by the amount of serine utilized with appropriate corrections for non-enzymatic serine utilization and conversion to pyruvate. The unit of cystathionine synthetase activity was defined as μ moles serine utilized/mg protein/60 minutes incubation. Protein was determined by the method of Lowry *et al.*(13).

Serine dehydrase activity was assayed by diluting 40% homogenates with KCl-EDTA to yield an 8% homogenate (wet weight/volume) and 1 ml of the diluted homogenate was added to the incubation mixture. The incubation mixture was identical with that used for assay of cystathionine synthetase except that DL-homocysteine was deleted. Total volume of reaction mixtures was 3 ml and incubation period was 20 minutes at 38°C

under 95% nitrogen 5% CO₂. The unit of serine dehydrase activity was defined as μ -moles pyruvate produced/mg protein/20 minutes incubation. Under conditions described, linear pyruvate formation was observed through 30 minutes incubation and protein concentrations of 4-6 mg/ml through 20 minutes. Cystathionine synthetase activity yielded linear reaction velocities for 90 minutes and a protein concentration of 10-14 mg/ml for 60 minutes incubation. Experiments in which the pH of both systems was varied indicated agreement with the reported optimum of 8.2-8.3(3).

Results. Serine dehydrase and cystathionine synthetase activities of rat liver, transplanted hepatomas and primary hepatomas were determined. The data are shown in Table I. None of the hepatomas, either transplanted or primary, had detectable amounts of either serine dehydrase or cystathionine synthetase activity. On the other hand, both enzyme activities were readily demonstrated in normal liver, livers from animals maintained on the semi-synthetic diet and liver tissue adjacent to primary lesions. When tumor homogenate was added to liver homogenate no significant alteration in either enzyme activity was observed. Furthermore, neither enzyme activity was activated in tumor homogenates by addition of liver homogenates or a boiled extract of liver homogenate.

Since the assay for cystathionine synthetase activity was dependent upon estimating the amount of serine utilized, further validation of cystathionine synthetase activity seemed advisable. When TCA extracts of incubation mixtures were subjected to paper chromatography(11) data as shown in Table II were obtained. Although liver homogenates consistently yielded evidence of cystathionine synthesis, no evidence of synthesis was observed with any of the transplanted hepatomas. Thus, these data (a) confirmed the previous observation that serine dehydrase was absent in the Novikoff hepatoma (2) and extended this observation to include other tumors of hepatic origin, and (b) showed that tissues lacking dehydrase activ-

[†] Sources were as follows: L-Serine, H. and M. Chemical Co.; Pyridoxal phosphate, Sigma Chemical Co.; DL-Homocysteine thiolactone, California Corporation for Biochemical Research. The thiolactone was converted to DL-homocysteine by adjusting an aqueous solution to pH 8.8 with 3 N NaOH just prior to its addition to the enzyme assay reaction mixture.

TABLE I. Serine Dehydrase and Cystathionine Synthetase Activities of Normal Rat Liver, Transplanted Hepatomas and Primary Hepatomas.

Tissue	Enzyme activity*			
		Serine dehydrase (units)		Cystathionine synthetase (units)
Liver†	(9)‡	1.01 ± .10§	(5)	.30 ± .05
"	(3)	.43 ± .04	(3)	.25 ± .18
Novikoff hepatoma	(3)	0	(4)	0
" " (ascites form)	(3)	0	(3)	0
3'-Me-DAB hepatoma	(3)	0	(3)	0
Primary "	(3)	0	(3)	0
Liver tissue adjacent to primary lesions	(3)	.52 ± .06	(3)	.28 ± .03

Conditions: For enzyme assays see text.

* Units: Serine dehydrase, μ moles pyruvic acid formed/mg protein/20 min.; cystathionine synthetase, μ moles serine utilized/mg protein/60 min.

† Animals maintained on Rockland chow diet.

‡ No. of experiments. For each experiment tissues from 2 to 3 animals were pooled.

§ Stand. dev.

|| Animals maintained on semi-synthetic, riboflavin-deficient diet for 12 wk.

ity also lacked cystathionine synthetase activity.

The absence of either serine dehydrase activity or cystathionine synthetase activity in primary hepatomas (Table I) indicated the loss of these enzyme activities during hepatocarcinogenesis with 3'-Me-DAB. Therefore, it was important to determine whether these losses occurred in precancerous liver. Activity of these enzymes in livers of ani-

mals fed 3'-Me-DAB for 0, 4, 8 and 12 weeks was compared to the activity of animals ingesting the basal diet (Table III). After 4 weeks on 3'-Me-DAB both serine dehydrase and cystathionine synthetase activity dropped sharply, however, by the 8th week activity had returned to normal or higher levels. At 12 weeks values approximated those observed in basal diet animals and values observed for liver tissue adjacent to primary lesions. Sub-

TABLE II. Paper Chromatography of Reaction Mixtures for Assay of Cystathionine synthetase Activity.*

			Position of ninhydrin positive compounds†	
			R _f values	
	L-cystathionine	(4)‡	.098 ± .003§	
	L-homocysteine	(2)		.197 ± .006
	L-serine	(3)		.203 ± .004
Reaction mixture for cystathionine synthetase activity with:				
Liver homogenate	No incubation	(2)		.205 ± .020
	60 min. incubation	(2)	.096 ± .019	.210 ± .025
Novikoff ascites homogenate	No incubation	(2)		.216 ± .008
	60 min. incubation	(2)		.216 ± .019
Novikoff homogenate	No incubation	(2)		.204 ± .021
	60 min. incubation	(4)		.205 ± .013
3'-Me-DAB hepatoma homogenate	No incubation	(2)		.204 ± .021
	60 min. incubation	(2)		.215 ± .020

* Solvent system, n-butanol:acetic acid:water (60:15:25).

† Color developed by .25% w/v ninhydrin in acetone.

‡ No. of experiments.

§ Stand. dev.

TABLE III. Comparison of Serine Dehydrase and Cystathionine Synthetase Activities of Rat Liver during Carcinogenesis with 3'-Methyl-4-dimethylaminoazobenzene.

Time, wk	Enzyme activity*			
	Serine dehydrase		Cystathionine synthetase	
	Basal	3'-Me-DAB	Basal	3'-Me-DAB
0	(3)† .60 ± .08‡		(3) .26 ± .09	(3)
4	(3) .42 ± .06	(3) .28 ± .12	(3) .16 ± .12	(3) .02 ± .41
8	(3) .40 ± .08	(3) .96 ± .27	(3) .19 ± .09	(3) .27 ± .12
12	(3) .43 ± .04	(3) .42 ± .03	(3) .25 ± .18	(3) .26 ± .04

Conditions: For enzyme assays see text. All animals were conditioned on basal diet 7 days prior to start of experiment. 3'-Me-DAB concentration was 0.06%.

* Units: Serine dehydrase, μ moles pyruvate formed/mg protein/20 min. incubation; cystathionine synthetase, μ moles serine utilized/mg protein/60 min. incubation.

† No. of experiments. For each experiment tissues from animals were pooled.

‡ Stand. dev.

sequent experiments in which 4'-fluoro-DAB†, DAB, 2'-methyl-DAB or 4'-methyl-DAB were fed to animals for 4 weeks, activity losses with both enzymes approximated those observed at 4 weeks with 3'-Me-DAB. Since these azo dyes differed markedly in their ability to induce lesions(14), it appeared that the early losses in both enzyme activities were not associated with carcinogenesis *per se*.

Throughout the present study, under conditions which led to alteration or loss of serine dehydrase activity, a similar alteration or loss of cystathionine activity was observed. Although these similarities could have been due to other factors, the data supported the contention that the conversion of serine to pyruvate and the condensation of serine and homocysteine to form cystathionine were reactions catalyzed by a single enzyme(3).

Since conversion of homocysteine to cysteine is mediated by cystathionine synthetase(3) and cystathionase(15), absence of cystathionine synthetase activity may account for the inability of homocysteine to replace cystine in the *in vitro* growth of the 3'-Me-DAB hepatoma(16). A similar inability to replace the cysteine requirement with homocysteine was reported for chick embryonic heart fibroblast cultures(17), however, Eagle (18) reported that in cultures of human cell strains, homocysteine was utilized for cysteine synthesis. It was subsequently shown (19) that the apparent homocysteine-cysteine interconversion was due to mobilization

of cysteine residues bound to the serum component of the medium.

Summary. Serine dehydrase and cystathionine synthetase activities were determined in rat liver, precancerous livers, primary hepatomas induced with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) and transplanted hepatomas induced with azo dyes. Both enzyme activities were absent in all malignant tissues assayed. After 4 weeks on diets containing 3'-Me-DAB both enzyme activities were reduced more than 50% but returned to normal values at 8 and 12 weeks. Precancerous alterations in these enzyme activities did not appear to be correlated with the carcinogenic activity of azo dyes employed. Deletion or alteration patterns in all tissues assayed supported the contention that both enzyme activities were manifested by a single apoenzyme.

1. Novikoff, A. B., *Cancer Research*, 1957, v17, 1010.
2. Auerbach, V. H., Waisman, H. A., *ibid.*, 1958, v18, 543.
3. Selim, A. S. M., Greenberg, D. M., *J. Biol. Chem.*, 1959, v234, 1474.
4. Potter, V. R., *U. Mich. Med. Bull.*, 1957, v23, 401.
5. ———, *Fed. Proc.*, 1960, v19, 312.
6. Chan, S. K., McCoy, T. A., Kizer, D. E., *Proc. Soc. Exp. Biol. and Med.*, 1959, v100, 420.
7. ———, *ibid.*, 1959, v102, 102.
8. Kizer, D. E., McCoy, T. A., *ibid.*, 1959, v102, 136.
9. Friedemann, T. E., Haugen, G. E., *J. Biol. Chem.*, 1943, v147, 415.

† DAB refers to 4-dimethylaminoazobenzene.

10. Frisell, W. R., Meech, L. A., Mackenzie, C. G., *ibid.*, 1954, v207, 709.
11. Woiwod, A. J., *J. Gen. Microbiol.*, 1949, v3, 312.
12. Toennies, G., Kolb, J. J., *Anal. Chem.*, 1951, v23, 823.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.
14. Miller, J. A., Miller, E. C., *Advances in Cancer Research*, vI, Academic Press, N. Y., 1953, p351.
15. Matsuo, Y., Greenberg, D. M., *J. Biol. Chem.*, 1959, v234, 507.
16. McCoy, T. A., Maxwell, M., *J. Nat. Cancer Inst.*, 1959, v23, 385.
17. Morgan, J. F., Morton, H. J., *J. Biol. Chem.*, 1955, v215, 539.
18. Eagle, H., *Bacteriol. Rev.*, 1958, v22, 217.
19. Eagle, H., Oyama, V. I., Piez, K. A., *J. Biol. Chem.*, 1960, v235, 1719.

Received January 23, 1961. P.S.E.B.M., 1961, v106.

Histamine Release into the Circulation by Meperidine (Demerol®).^{*} (26477)

ROBERT ZEPPA,[†] DORIS C. GROSSEKREUTZ AND KENNETH SUGIOKA
(Introduced by N. A. Womack)

*Department of Surgery, School of Medicine, University of North Carolina and North Carolina
Memorial Hospital, Chapel Hill*

Hypotension is the primary cardiovascular effect of meperidine. This pharmacologic action has been demonstrated in the dog with various intravenous doses, both small and large. This fall in blood pressure has been attributed to a number of mechanisms, including vasodilatation by direct action, central vasomotor depression and perhaps, ganglionic blockade. Schacter(1) measured increases in free histamine in cat muscle and skin after treatment with meperidine. Subsequently, the possibility that histamine release contributes in part to the mechanism of meperidine induced hypotension has been suggested by several workers(2,3). Since there is no quantitative information concerning this effect in the intact dog, this study was done to find out if this drug is a histamine releasing agent in the dog and if so, whether enough histamine is released to cause hypotension.

Methods. Anesthetized (pentobarbital, 35 mg/kg) mongrel dogs received artificial ventilation through a cuffed endotracheal tube by means of a Starling pump. Blood pressure was recorded continuously from a catheter in the femoral artery connected to a Statham pressure transducer. The tracing

was obtained on an Offner direct writing recorder. After a steady state was obtained, meperidine 5 mg/kg of a 10% solution, was injected rapidly into the femoral vein, followed with 1 ml of isotonic saline. Prior to and at one, 3 and 5 minutes after injection, samples of venous blood were taken into siliconized syringes containing 1:100 of 10% disodiummethylenediaminetetraacetate. Histamine was measured by the method of Shore and co-workers(4) with one modification; a Farrand photofluorometer, model A was employed instead of a spectrophotofluorometer. The approximate activating and fluorescent wave lengths were isolated by use of Corning glass filters, primary no. 5860 and secondary no. 3389. The sacrifice of some selectivity was replaced by the gain in ease of measuring duplicate specimens. Quenching occurred at a lower final concentration of histamine but the latter was proportional to fluorescence over the range .005 $\mu\text{g/ml}$ to .165 $\mu\text{g/ml}$. All measurements were performed on duplicate specimens. Three dogs received intravenous injections of histamine, .03 mg/kg. The dose was calculated from the average increase in blood histamine found at the one minute interval after meperidine injection in 5 dogs. Blood histamine was measured in only one of the 3 dogs.

^{*} This investigation supported by USPHS Grant.

[†] Markle Scholar in Medical Science.

TABLE I.

Dog wt, kg	Blood histamine, $\mu\text{g/ml}$, after meperidine, 5 mg/kg I.V.				Blood pressure after meperidine, 5 mg/kg I.V.			
	Control	1 min.	3 min.	5 min.	Control	1 min.	3 min.	5 min.
1-15	.12	.57	.30	.27	174/126	40/18	34/12	42/18
2-16	.18	.61	.54	.29	182/120	48/24	42/24	60/30
3-14	.12	.45	.43	.37	162/94	27/12	27/18	36/25
4-15	.06	.39	.25	.18	182/126	48/28	54/32	66/42
5-13	.05	.53	.41	.37	162/108	50/28	42/24	48/30
Avg*	.11 \pm .06	.51 \pm .11	.39 \pm .14	.30 \pm .10	172/115	43/22	40/22	50/29
6-13†	.06	.76	.12	.08	174/115	54/30	90/64	130/100

Comparisons of significance of change between control and post-meperidine averages reveal $p < .001$.

* \pm includes 95% confidence interval.

† Received histamine .03 mg/kg I.V.

Results. Rapid injection of meperidine into the circulation is followed by a marked lowering of arterial pressure and a rise in concentration of blood histamine to approximately 5 times control level (Table I). Mean concentration of blood histamine at control and post injection intervals varied between 0.11 $\mu\text{g/ml}$ prior to injection and 0.30 $\mu\text{g/ml}$ after 5 minutes. The largest increase occurred at the one minute period and the average was 0.51 $\mu\text{g/ml}$. The differences between control and post-injection values for histamine are significant, p being much lower than .001 for the 3 comparisons.

The temporal relationships between these changes are illustrated in Fig. 1. The nadir of blood pressure depression occurs during the interval of maximal velocity of histamine release.

Comparative tracings of the occurrence of hypotension after meperidine and histamine injections are seen in Fig. 2. Blood pressure falls more precipitously after histamine but recovers more rapidly. This is a reproduction of the tracing reported from Dog 6 (Table I). The 3 recordings from the dogs

receiving histamine were virtually identical.

Discussion. Assuming an average blood volume in the dog of 75 ml/kg, a conservative estimate(5), then the change in amount of circulating histamine induced in one minute by administration of meperidine is equivalent to .03 mg/kg of histamine base. This is sufficient to produce the peripheral vasodilatation and hypotension commonly attributed to meperidine *per se* (Fig. 2). In addition

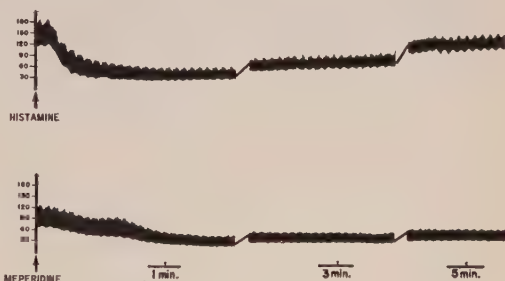


FIG. 2. Effects of histamine and meperidine on blood pressure.

the velocity of change in both parameters occurs simultaneously (Fig. 1), thus the temporal as well as quantitative requirements of such a relationship are satisfied. These conclusions are at some variance with those of Schacter(1), who reported that the ability of meperidine to release histamine is a property incidental to its major pharmacologic actions. While such deductions may be valid from observations in the eviscerated cat, they cannot be applied indiscriminately to all species. For example, the ability of meperidine to reverse histamine induced bronchial spasm in the isolated and perfused guinea pig lung is not sufficient evidence to classify the drug as a gen-

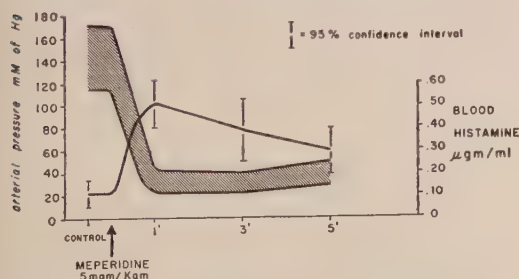


FIG. 1. Time relationship of blood histamine and blood pressure changes (avg from 5 dogs).

eral anti-histaminic agent. In the anesthetized dog, the major cardiovascular effects of meperidine appear to be mediated through release of histamine, although a combined effect of meperidine and histamine on the peripheral vascular system is not ruled out. Administration of anti-histaminic drugs in large doses has failed to separate these effects and other means are being considered to settle this point.

The differences in configuration of the tracings illustrating the effects of histamine and meperidine (Fig. 2) probably have two causes. First, the initial drop in the histamine tracing is more precipitous because more histamine is perfused per unit of time. These animals received the amine in one or 2 seconds whereas the meperidine group released this amount of endogenous histamine over a one minute period. The return to normal is more rapid in histamine treated ani-

mals because there is no prolonged release of the amine. This is evident by the rapid return of the blood values to control levels (Table 1).

Summary. Meperidine is a potent histamine releasing agent in the anesthetized dog. The quantitative and kinetic aspects of this phenomenon strongly suggest that it is responsible for the cardiovascular effects of this drug.

1. Schacter, M., *Brit. J. Pharmacol.*, 1952, v7, 646.
2. Sugioka, K., Boniface, K. J., Davis, D. A., *Anesthesiology*, 1957, v8, 623.
3. Goodman, L. S., Gilman, A., *The Pharmacologic Basis of Therapeutics*, 2nd ed., 1958, MacMillan Co., New York, 263.
4. Shore, P. A., Burkhalter, A., Cohn, V. H., Jr., *J. Pharm. Exp. Ther.*, 1959, v127, 182.
5. Von Porat, B., *Acta Med. Scand.*, 1951, v140, suppl. 256.

Received January 24, 1961. P.S.E.B.M., 1961, v106.

Employment of Polyethylene Tubing for Production of Intra-Arterial Thrombi in Rabbits and Rats.* (26478)

MEYER FRIEDMAN AND SANFORD O. BYERS

Harold Brunn Institute, Mount Zion Hospital & Medical Center, San Francisco, Calif.

Recently we have described(1) a simple method of inducing thrombi of approximately uniform size and weight in both the arteries and veins of rabbits and rats. This technique consisted of insertion into the lumen of a vessel of a small coil of Mg-Al wire previously treated with $ZnCl_2$. The thrombi form within a few hours, apparently triggered by the rapid electrolytic dissociation of the coil.

Other methods were sought for inducing intravascular thrombosis in an intact vessel without interrupting flow of blood, however, because a less fulminant, slower forming thrombus might offer certain advantages in various experimental studies. The present report describes a new method which is simple, relatively reliable and produces thrombi of

approximately uniform size, weight and content.

Methods. It was discovered accidentally that small segments of polyethylene tubing[†] will serve as a nidus for thrombus formation. Further studies indicated that if these segments of tubing are split lengthwise and the resulting gutter-like segments inserted, thrombus formation almost invariably occurs within 72 hours in the trough of the segments.

Accordingly, 30 young male rabbits were anesthetized with pentobarbital and their aortae isolated below the exit of the renal arteries. Each aorta was gently occluded by traction upon ligatures approximately 2 cm apart and a very small incision made. Through this incision was inserted a 0.5 inch segment of polyethylene tubing (i.d. = 0.045" and

* Aided by grants from San Francisco Heart Assn., Sacramento Heart Assn., Contra Costa County Heart Assn. and Nat. Inst. of Health, Nat. Heart Inst.

[†] Clay-Adams PE-160, Intramedic (Medical Formulation PHF) polyethylene tubing.

o.d. = 0.062") cut in half lengthwise. Insertion was manipulated so that the cut walls of the semicircular section of the polyethylene segment were usually in close contact with the intimal surface of the aorta and the convex portion of the segment lay free in the lumen. When this is done, the thrombus forming later is directly in contact with the intima. A silk thread carried at one end of the polyethylene segment served as an anchor. The aortic incision was closed by a single, previously placed mattress type of suture.

Essentially the same procedure was followed with 10 rats except that the tubing employed was smaller in diameter (i.d. = 0.023" and o.d. = 0.038") and shorter (0.25").

Rabbits were sacrificed either at 72 hours, 3 weeks or 10 weeks. All rats were sacrificed at 72 hours. The thrombotic processes found in the aorta were examined grossly and microscopically.

Results. The rabbits tolerated insertion of polyethylene segments as well as other rabbits had tolerated insertion of Mg-Al coils (1). Thus 26 of the 30 rabbits survived the process of insertion itself. The remaining 4 died exhibiting paralysis of the hind legs immediately subsequent to surgery. All of the 10 rats survived the insertion.

Nine rabbits and all 10 rats were sacrificed 72 hours after insertion of tubing. A rather firm thrombus was found at site of operation in 7 of the 9 rabbits and in 8 of the 10 rats. It usually occupied the entire trough of the tubing. The gross appearance of the thrombi in rabbits and rats was identical except for the difference in size. In most cases (Fig. 1), this thrombus was almost pure white, but occasionally small areas obviously rich in red blood cells were scattered here and there in the thrombus. At 72 hours, thrombi of both species were more firmly attached to the wall of the tubing than to the intima of the vessel.

On microscopic examination, the 72 hour thrombi of both species appeared identical. They were composed of an amorphous almost acellular structure interlaced with fibrin strands, (as identified by Mallory's Phosphotungstic Acid Hematoxylin Stain). In

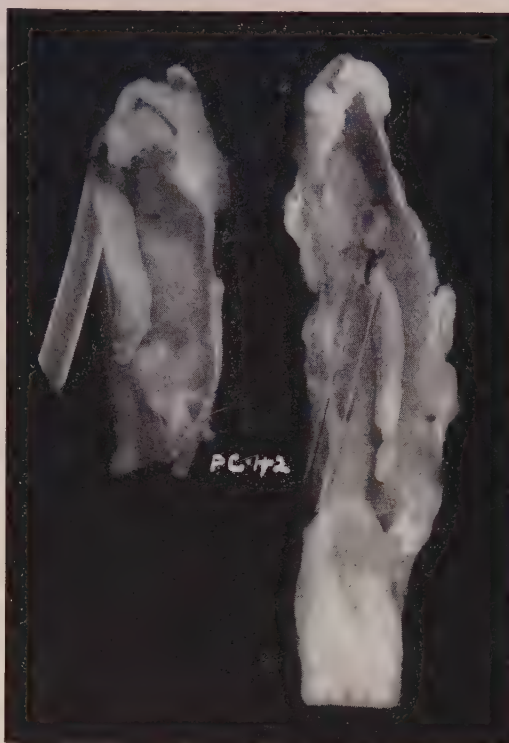


FIG. 1. Two rat aortas, 72 hr after insertion of polyethylene tubing. The thrombus in each aorta has been freed from the trough of the tubing. Note the white rather solid appearance of both thrombi and lack of attachment to the aorta. Silk thread anchoring the segment of tubing can be seen in aorta on right.

some sections, scattered clumps of red and white blood cells were observed. No cellular infiltration from the aortic intima was observed at this time.

Eight of the 10 rabbits sacrificed at 21 days exhibited thrombus formation in the space between the concave surface of the polyethylene tubing and wall of the aorta. The thrombi were adherent to the vessel walls and gross intimal reactions were easily discerned. Also, clumps of cells were beginning to extend over the entire length of polyethylene tubing (Fig. 2). On microscopic examination (Fig. 3), the interior of the thrombus was found to resemble closely the 72 hour thrombus. In the 21 day thrombi, however, proliferating intimal cells could be discerned extending from the intimal tissue directly beneath the thrombus. New small blood vessels also were observed accompany-



FIG. 2. Two rabbit aortas, 21 days after insertion of tubing. Tubing has been removed from aorta on the left and lifted from aorta on the right. A superficial post mortem film of fresh clot rich in red blood cells is attached to the primary thrombus in aorta IE-10. In aorta IE-G, the new intimal growth had begun to ascend the external convex sides of the tubing. Both thrombi are now firmly attached to wall of aorta.

ing the proliferating intimal tissue. That part of the media directly underlying the thrombus also usually showed partial fragmentation and disorganization. In none of the thrombi was there an evidence of past or present inflammatory processes.

In the 9 rabbits sacrificed at 10 weeks, the polyethylene segment was found to be totally covered by newly formed tissue. This covering tissue was incised and in 7 of the 9 rabbits the troughs were filled by rather firm tissue of pinkish white color (Fig. 4.) In the remaining 2 rabbits, the tubing troughs had faced the luminal flow of blood. Nevertheless, the tissue in these latter 2 was identical with that observed in the others.

Microscopic examination of these latter 2 plaques (Fig. 5) revealed that the original thrombus had been totally replaced by liv-

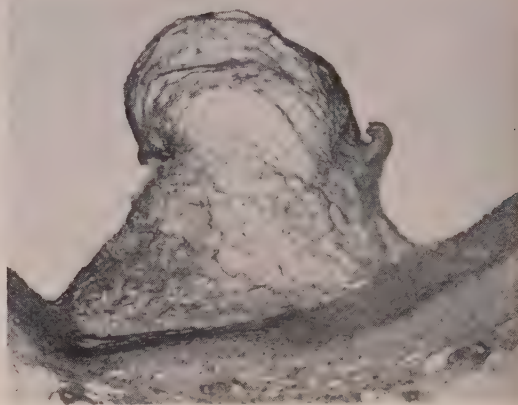


FIG. 3. Low power view of thrombotic process in the rabbit aorta ($\times 22$) 21 days after insertion of tubing (PTAH stain). The rather dense structure of the thrombus with few cells and a few interlacing strands of fibrin is shown. The newly formed intimal tissue beginning to ascend the lateral walls of the thrombus also can be seen. Note thinning and partial disorganization of that portion of the media immediately subjacent to thrombus.

ing tissue newly derived from the intima of the aorta. No histological differences were observed between the plaques regardless of the orientation of the tubing within the aortic lumen.

Discussion. The desirability of inducing thrombi in the arterial system has been intensified ever since Duguid(2) revived Rokitsky's concepts(3) concerning the possible origin of atherosclerotic plaques. Most of the various methods that have been employed in inducing thrombi (4,5,6,7,8,9,10, 11) have consisted either of injecting already formed thrombi into the venous system or of inducing massive thrombosis in a totally occluded arterial segment. Such methods, however, are not suitable for experiments concerned with the possible relationship between thrombus formation and atherosclerosis(2) because the thrombi induced by most methods either form in the venous system or in an artery whose flow has been totally obstructed by the thrombus itself. Stone and Lord(12) observed that Mg-Al wire induced thrombi in the rapidly flowing blood of arteries. This method as modified by us(1) appeared to offer a very satisfactory technique for study of arterial thrombus formation and subsequent plaque placement. The



FIG. 4. Aorta of rabbit 10 wk after insertion of tubing. Tissue covering the tubing was incised and tubing removed for photography. Although the central mass exhibits the same external form as thrombi in Fig. 1 and 2, it is no longer a fresh thrombus but has been organized and replaced by fibrous tissue. A small and superficial postmortem film of fresh clot is attached to lower half of plaque. Note lateral prolongations of intimal tissue that had covered external surface of the tubing.

presently described method offers a second technique by which arterial thrombi may be regularly induced and their transformation into plaques observed at all stages. The aortic plaques resulting both from introduction of polyethylene tubing and insertion of Mg-Al coil bear a very close resemblance to the pearly white plaques observed in the human aorta.

Summary. Arterial thrombi were produced in the aortae of rabbits and rats by insertion of specially fashioned polyethylene tube seg-

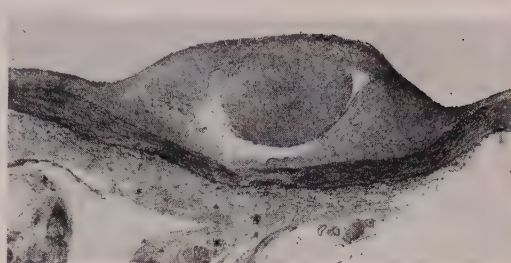


FIG. 5. Low power view of thrombotic process in rabbit aorta ($\times 16$) 10 wk after insertion of tubing (H and E stain). In this animal, the trough of the tubing had faced the lumen rather than wall of aorta. The thrombus within the trough has been completely organized by intimal tissue that had ascended the walls of tubing, then had descended into its trough. The empty space separating the central mass from walls of plaque was produced by the tubing removed prior to section and staining. Note thinning and partial disorganization of media beneath site of tubing.

ments. These thrombi differ from clots formed in static blood in that they are relatively acellular precipitates containing fibrin and probably platelets. These thrombi eventually are organized and lead to plaques in which all elements of the initial thrombus are replaced by tissue from the intima. The resemblance of these plaques to the pearly white plaque frequently seen in the human aorta is striking.

1. Friedman, M., Byers, S. O., Pearl, F., *Am. J. Physiol.*, 1960, v199, 770.
2. Duguid, J. B., *J. Path. and Bact.*, 1948, v60, 57.
3. Rokitsansky, C., *A Manual of Pathological Anatomy*, Sydenham Soc., London, 1852, p261.
4. Murray, G. W. D., Jaques, L. B., Perrett, T. S., Best, C. H., *Surgery*, 1937, v2, 163.
5. Sawyer, P. N., Pate, J. W., Weldon, C. S., *Am. J. Physiol.* 1953, v175, 108.
6. Buermann, O., Blum, A. S., *J. Lab. and Clin. Med.*, 1959, v53, 258.
7. Schwartz, S. I., *Surg. Gynec. and Obst.*, 1959, v108, 533.
8. Copley, A. L., Stefko, P. L., *Surg. Gynec. and Obst.*, 1947, v84, 451.
9. Bayles, P. W., *Am. J. Clin. Path.*, 1958, v30, 423.
10. Filshie, I., Scott, G. B. D., *J. Path. and Bact.*, 1958, v76, 71.
11. Wessler, S., Reiner, L., Freiman, D. G., Reiner, S. M., Lertzman, M., *Circ.*, 1959, v20, 864.
12. Stone, P., Lord, W. J., Jr., *Surgery*, 1951, v30, 987.

Received January 27, 1961. P.S.E.B.M., 1961, v106.

Cholesterol Content of Human Liver After Feeding of Corn Oil and Hydrogenated Coconut Oil.* (26479)

IVAN D. FRANTZ, JR. AND JAMES B. CAREY, JR.

Departments of Medicine and Physiological Chemistry, Medical School, University of Minnesota, Minneapolis

The mechanism by which diets rich in unsaturated fats cause reduction of the serum cholesterol in human beings is not clear. It has been proposed that the reduction comes about through increased excretion of the total products of cholesterol catabolism(1), increased excretion of bile acids(2), or decreased synthesis of cholesterol(3). Another possibility, made plausible by observations in animals(4,5), is that cholesterol is shifted from the serum to the tissues. Avigan and Steinberg(5) found, for example, that rats fed corn oil showed a large rise in liver cholesterol, while rats fed coconut oil showed no significant change. Although the serum cholesterol in the animals fed corn oil was slightly lower than in those fed coconut oil, it was not lower than in the animals on a control diet, in contrast to the situation in man. Nevertheless, the hypothesis that unsaturated fats cause a shift of cholesterol from blood to liver seemed worth testing in human beings.

Methods. Twelve healthy male subjects with high normal levels of serum cholesterol were chosen from more than one hundred volunteers. The men were divided into 2 groups of 6 men each. Both groups were allowed to eat their institutional, American type diet *ad libitum*. Each man in Group I received, in addition, one ounce of hydrogenated coconut oil before each meal. Each man in Group II received a similar ration of corn oil. The experimental period was one month. Serum cholesterol was measured twice at the beginning and twice at the end of experiment, by the Abell method(6). A punch biopsy of the liver was obtained at beginning and end. In most instances 15-20 mg of wet liver were available for cholesterol analysis, which was done by a micro modification of the Abell method. The tissue specimens were first ho-

mogenized in 1 ml of water. Duplicate 0.05 ml aliquots were taken for protein nitrogen analysis by the method of Nayyar and Glick (7). Duplicate 0.2 ml aliquots were hydrolyzed and partitioned against 4 ml of petroleum ether, as in the Abell procedure for serum. Three ml portions of the petroleum ether extracts were evaporated to dryness in 12 ml conical centrifuge tubes. The residue was dissolved in 0.15 ml of Liebermann-Burchard color reagent, and 0.12 ml was removed for measurement of the optical density in microcuvettes in the Beckman DU spectrophotometer. The standard error of values obtained by this technic was 7%. Cholesterol concentrations were calculated in mg/mg of protein nitrogen, but are reported below in the more familiar units of mg/100 g of wet tissue, on the basis of a conversion factor obtained by analysis of macro specimens of normal human liver for protein nitrogen.

Results and discussion. Table I shows body weight, serum cholesterol concentration, and liver cholesterol concentration at beginning and end of experimental period for each subject. Body weights changed very little, in spite of the high caloric content of the dietary supplements. This failure of the subjects to gain weight is probably attributable to the fact that the oil was given just before meals, with an adverse effect on their appetites. Serum cholesterol concentrations after corn oil feeding dropped in all cases. Liver cholesterol concentrations also dropped, and the decrease was significant at the 0.05 level. In view of this finding, it appears unlikely that the fall in serum cholesterol produced by corn oil feeding in man is due to a shift of cholesterol from the blood to the liver.

Summary. Three ounces of corn oil daily were added to the diets of 6 men for a period of one month. Three ounces of hydrogenated coconut oil daily were added to the diets of a similar group of controls. Serum cholesterol

* Supported by grants from Life Insurance Medical Research Fund and from Nat. Heart Inst., U.S.P.H.S.

TABLE I. Effect of Corn Oil and Hydrogenated Coconut Oil on Body Weight, Serum Cholesterol, and Liver Cholesterol.

Subject	Diet	Body wt			Serum cholesterol			Liver cholesterol		
		Initial, lb	Final, lb	% change	Initial, mg %	Final, mg %	% change	Initial mg/100 g wet tissue	Final	% change
1	Hydrog.	179½	180½	+ .6	261	301	+15	281	282	0
2	coconut	216¼	213½	-1.3	305	329	+ 8	343	315	- 8
3	oil	170½	167½	-1.8	316	316	0	1459	1218	-17
4		181½	180	- .8	267	278	+ 4	428	559	+31
5		143½	142½	- .7	280	264	- 6	353	534	+51
6		172	175	+1.7	265	273	+ 3	319	307	- 4
	Avg			- .4			+ 4*			+ 9*
7	Corn oil	219¾	215	-2.2	279	260	- 7	460	310	-33
8		172½	171	- .9	312	266	-18	910	545	-40
9		163¾	161	-1.7	294	272	- 7	331	349	+ 5
10		201¼	204	+1.4	272	245	-10	558	382	-32
11		204¼	204	- .1	325	298	- 8	531	410	-23
12		183¼	183½	+ .1	266	251	- 6	†	763	†
	Avg			- .6			- 9†			-25†

* Change from initial value not statistically significant.

† " " " " " probably significant ($p < 0.05$).

‡ Although cholesterol was present, this specimen contained no detectable protein by the method used. It is assumed that the biopsy represented fat, rather than liver parenchyma.

concentration fell an average of 9% in the men fed corn oil, but did not change significantly in the controls. Liver cholesterol concentration, as measured by liver biopsy, fell an average of 25% in the men fed corn oil. No consistent effect was observed in the controls. It is concluded that the fall in serum cholesterol produced by corn oil feeding in man is probably not due to a shift of cholesterol from the blood to the liver.

1. Hellman, L., Rosenfeld, R. S., Insull, W., Jr., Ahrens, E. H., Jr., *J. Clin. Invest.*, 1957, v36, 898.

2. Gordon, H., Lewis, B., Eales, L., Brock, J. F., *Lancet*, 1957, v273, 1299.

3. Kinsell, L. W., Michaels, G. D., Walker, G., Conklin, J., *Circ.*, 1960, v22, 661.

4. Alfin-Slater, R. B., Schotz, M. C., Shimoda, F., Deuel, H. J., Jr., *J. Biol. Chem.*, 1952, v195, 311.

5. Avigan, J., Steinberg, D., *Proc. Soc. Exp. Biol. and Med.*, 1958, v97, 814.

6. Abell, L. L., Levy, B. B., Brodie, B. B., Kendall, F. E., *J. Biol. Chem.*, 1952, v195, 357.

7. Nayyar, S. N., Glick, D., *J. Histochem.*, 1954, v2, 282.

Received January 30, 1961. P.S.E.B.M., 1961, v106.

A Modified Plastic Petri Dish for Cell and Tissue Cultures.* (26480)

WILLIAM G. COOPER† (Introduced by Donn L. Smith)

Department of Anatomy, University of Colorado School of Medicine, Denver

For many years the 60 mm glass Petri dish has served as a standard culture vessel for maintenance of a variety of cell strains and tissue explants *in vitro*. Monolayer cultures have been maintained on the inside bottom of the Petri dish overlaid by a prescribed vol-

ume of liquid medium whose pH is maintained by the humidified 5% CO₂ environment of the incubator in which the culture dishes are placed. Observations of the growing cultures require an inverted microscope or replacement of the dish top with a device enabling one to bring the objective close enough so that the cells can be viewed with an ordinary microscope. Both systems leave

* Supported by grants from Life Insurance Medical Research Fund and U.S.P.H.S.

† Senior Research Fellow, U.S.P.H.S. (SF-332)



FIG. 1. A. Complete dish, assembled. B. Recessed top. C. Dish bottom.

much to be desired regarding the cytological resolution obtained. The culture dish herein described has many features which tend to overcome some of the problems encountered in growing monolayer cultures in conventional dishes.

The culture dish[†] is made of a pre-sterilized polystyrene plastic. The lid has a sharply recessed area 40 mm in diameter (Fig. 1, B). The sides of the bottom (Fig. 1, C) of the dish are tapered to facilitate their stacking or filing for maintaining permanent records of experiments. The optical working distance from top of the lid to inside bottom of the dish (cell surface) is 2.6 mm. This enables one to study individual cells of the monolayer by using an ordinary light microscope or phase microscope (Fig. 2). When making observations with an inverted microscope (Fig. 3), the recessed lid permits the phase condenser to be focused on the cell surface, thereby producing much better definition of cell structure at higher magnifications. All of these optical procedures do not increase the chance of contamination since all are done with the recessed lid in place.

Despite the presence of the recessed lid, there is ample space between the lid and the bottom to allow for maintenance of the proper pH of the medium by the CO₂ environment of the incubator. Approximately 4 ml of medium will flood the bottom of the dish and eliminate the air interface between lid and medium. The maximum working

volume of medium is approximately 7 ml.

This dish has been utilized in conjunction with time-lapse cinephotomicrographic techniques to record the process of trypsinization in various cell strains and tissues (Fig. 3). Additional experiments involving the applicability of this dish to long term time-lapse cinephotomicrographic experiments are now in progress.

Many histochemical procedures can be carried out completely within this dish. Where the experimental procedure requires an air tight environment, the dish can be sealed by rimming the bottom of the dish



FIG. 2. Photomicrograph of a monolayer culture of L-929 cell strain growing on inner surface of plastic petri dish bottom. The field was photographed on 16 mm Gaevapan movie film from above, through the recessed top, utilizing a 10× A.O. Dark M. phase objective.

FIG. 3. Photomicrograph of a monolayer culture of L-929 cell strain growing on inner surface of plastic petri dish bottom. The field was photographed on 16 mm Gaevapan movie film with the aid of a Zeiss Plankton microscope and a 40× Zeiss phase contrast objective.

[†] Currently available from Falcon Plastics, Los Angeles, Calif.

with silicone or a nontoxic plastic cement.

The design of this dish should aid the investigator in obtaining better cytological detail of monolayer cultures without increasing the danger of contamination and should be especially useful in laboratories where fre-

quent screening is required of large numbers of plates as well as in conducting student laboratory experiments where the supply of inverted microscopes is inadequate.

Received February 16, 1961. P.S.E.B.M., 1961, v106.

Effect of Glutamate and Glycine on Cock Sperm Metabolism. (26481)

S. EL ZAYAT AND A. VAN TIENHOVEN (Introduced by M. L. Scott)

Agricultural Experiment Station, Cornell University, Ithaca, N. Y.

During investigations on the effect of chloride on cock sperm metabolism it was found that replacement of chloride by glutamate in a Tyrode diluent resulted in a linear depression of the rate of fructolysis and of respiration rate(1). Replacement of chloride by phosphate did not result in a significant change in rate of metabolism. The present paper reports results obtained in a further investigation of these phenomena.

Materials and methods. The methods used to obtain semen, to measure respiration rate and utilization of fructolysis have been previously described(1). In all experiments a 1:10 dilution rate was used. All experiments were designed as completely randomized blocks and analyzed according to the methods outlined by Snedecor(2). Diluents were made isotonic to seminal plasma ($\Delta = 0.62^\circ\text{C}$). Freezing points were determined with the aid of a Fiske milkcryoscope. Compositions of the basic diluents employed are given in Table I. A designation of 25% "phosphate" means that 3 parts of "glutamate" diluent were mixed with 1

part of "complete phosphate" diluent. All diluents contained 100 mg dihydro streptomycin and 10 mg tetracycline hydrochloride per 100 ml(3). To determine the catabolism of glutamate 6 μC of glutamate 1-C^{14} were added per Warburg flask; a similar procedure was used in the case of glycine. The C^{14}O_2 was trapped in KOH and either converted to $\text{BaC}^{14}\text{O}_3$ and the radioactivity of $\text{BaC}^{14}\text{O}_3$ determined, or the KOH and $\text{KHC}^{14}\text{O}_3$ were plated directly on in a small metal cup and radioactivity measured on the dried material. Radioactivity was measured with a thin mica window Geiger-Müller counter.

Experiments and results. The effect of replacement of chloride by phosphate or glutamate was investigated in an experiment in which the 3 diluents were used simultaneously. The results are presented in Table II. Analysis of variance revealed: 1) Respiration rates of Tyrode-diluted and phosphate-diluted fowl semen are higher ($P < 0.001$) than that of glutamate-diluted semen; 2) Diluents had no significant ($P > 0.20$) effect on rate of fructolysis. 3) The interaction be-

TABLE I. Composition of Diluents Used in Fowl Semen Experiments.

Isotonic solution	g/100 ml	"Complete phosphate"	Tyrode	"Glutamate"
		ml		
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2.04	1.00	1.00	1.00
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	2.48	.40	.40	.40
KCl	1.25	1.60	1.60	1.60
Na H CO_3	1.53	6.50	6.50	6.50
Fructose	6.00	4.00	4.00	4.00
Na monoglutamate	3.21	—	—	86.50
Phosphate buffer*	—	86.50	—	—
Na Cl	.97	—	86.50	—

* 16.34 g Na_2HPO_4 plus 5.16 g NaH_2PO_4 in 1000 cc water.

TABLE II. Effect of Phosphate and Glutamate Replacement of Chloride in Tyrode Diluent on Metabolic Rates of Fowl Semen.

Diluent	Respiration rate		Fructose used, $\mu\text{g}/10^8/2 \text{ hr}$
	$\mu\text{l O}_2/10^8/1 \text{ hr}$	$\mu\text{l O}_2/10^8/2 \text{ hr}$	
Tyrode	4.11	6.95	62.47
Complete phosphate	4.22	6.11	13.73
Glutamate	3.39	5.37	17.76
N	12	12	6

tween duration of incubation and diluents was significant ($P < 0.05$). The interactions (Tyrode versus Phosphate + Glutamate) \times duration of incubation were significant ($P < 0.05$) but the comparison (Phosphate *vs.* Glutamate) \times duration of incubation was not ($P > 0.20$). These interactions suggested that phosphate might have no effect on respiration rate during the first hour of incubation but that it might depress respiration later in the incubation period. Such an effect would be similar to that obtained by addition of glycine to a Tyrode diluent(4).

To investigate this problem further we incubated semen diluted with the 3 diluents, mentioned above, for 6 hours. Hourly respiration rates observed in these experiments are given in Table III together with correlation and regression coefficients of hourly respiration rate on time. To make comparisons with previously published data on glycine we recalculated the coefficients for the first

TABLE III. Hourly Respiration Rates for Fowl Semen Diluted with 3 Different Diluents and Calculated Regression (b) and Correlation Coefficients (r) of These Respiration Rates on Time of Incubation.

Hr	Tyrode	Complete phosphate		Glutamate		
	$\mu\text{l O}_2/10^8/\text{hr}$					
1	3.85	3.99		2.90		
2	2.46	1.49		1.60		
3	2.77	2.49		2.27		
4	1.75	1.59		1.53		
5	1.96	2.06		2.31		
6	1.90	2.19		1.42		
Diluent		N	b	P	r	P
Tyrode		6	.3508	<.05	.735	>.05
Complete phosphate		6	.2337	>.10	.482	>.05
Glutamate		6	.1714	>.10	.552	>.05
Tyrode*		3	.3514	<.05	.837	>.05
Tyrode - glycine*		3	.1420	<.01	.925	<.01

* See text.

6 hour period in those experiments. These data show: 1) A remarkable agreement in regression coefficients obtained with Tyrode diluent in 2 different experiments. 2) Variations in hourly respiration rate of semen diluted with glutamate and phosphate diluent showed no significant relationship with duration of incubation. We may conclude from this that either there was no relationship and thus the variations were random, indicating that the lines were horizontal, or that the regressions although real were not significant because not enough points were available to demonstrate this relationship. In either case we may conclude that the regression lines for Tyrode-diluted semen and those for phosphate-diluted and glutamate-diluted semen will cross. This in turn means that phosphate or glutamate prevents the gradual decrease in respiration rate observed with Tyrode-diluted semen.

These experiments suggested 2 further questions: 1) what is the effect of graded replacement of phosphate by glutamate, in the complete phosphate diluent, on metabolic rates? 2) Are glutamate and/or glycine catabolized by the fowl semen?

Results of investigations of the first question are given in Table IV. Analysis of variance showed that: 1) The quadratic components of the effect of phosphate replacement by glutamate were significant ($P < 0.01$). 2) Differences in amounts of fructose used were not significant ($P > 0.10$).

These data thus indicated that there was an optimum ratio of phosphate and glutamate with respect to respiration rate, and that this optimum lies near the 50:50 ratio. The significance of the apparent discrepancies between data of Table II and Table IV is discussed below.

Data concerning the second question raised above are summarized in Table V. These results demonstrate: 1) glutamate depresses rate of fructolysis under aerobic and anaerobic conditions; 2) glutamate depresses respiration rate; 3) glycine depresses respiration rate; 4) glutamate is catabolized to a limited extent by fowl semen; 5) rate of catabolism of glycine by fowl semen is negligible; 6) analysis of variance showed that the dif-

TABLE IV. Effect of Graded Levels of Glutamate and Phosphate on Metabolic Rates of Fowl Semen.

Diluent	Respiration rate		Fructose used, $\mu\text{g}/10^8/2 \text{ hr}$
	$\mu\text{l O}_2/10^8/1 \text{ hr}$	$\mu\text{l O}_2/10^8/2 \text{ hr}$	
Phosphate			
100%	3.06	5.21	24.47
75%	3.13	5.25	29.39
50%	3.39	5.48	25.16
25%	3.26	5.49	26.13
Glutamate			
100%	2.81	5.15	23.10
N	7	7	4

ference in fructose used under aerobic and anaerobic conditions was not significant ($P > 0.05$).

These results show that glutamate, in contrast with glycine, can be catabolized by fowl semen. With respect to glycine fowl sperm resemble sea-urchin sperm(5) but differ from bovine sperm(6,7). We tried to approximate whether the catabolism of glutamic acid might account for the smaller amount of fructose used under aerobic conditions. In these calculations we assumed that all fructose which disappeared had been metabolized to lactic acid(4) yielding 2 moles ATP for each mole of fructose used and we assumed that 36 moles of ATP would be generated per mole of glutamate catabolized to CO_2 . Based on these assumptions we made the following calculations: ATP generated in Tyrode diluent from fructolysis 2.678 micro moles; ATP generated in glutamate diluent: 0.165 micro moles from fructolysis plus 1.494 micro moles from glutamate thus yielding 1.65 g micro moles. Thus approximately 1 micro mole of ATP less is generated in the glutamate diluent. This calculation does not take account of differences in respiration rates in the 2 diluents since those differences may be due

to chelating effects of glutamate. The data obtained under anaerobic conditions show clearly that glutamate has an inhibitory effect on fructolysis even when glutamate cannot be catabolized. Under these conditions about 0.7 micro moles less of ATP are generated in the glutamate than in the Tyrode diluent.

Discussion. The results showing that chloride replacement by phosphate in a Tyrode diluent had no significant effect on respiratory or fructolytic rate of fowl sperm agree with previously obtained results(1). The inhibitory effect of glutamate on respiration also agrees with these results. Comparisons of the means obtained given in Tables II and V, and in Table IV, show that the magnitude of the difference in respiration rate between phosphate-diluted and glutamate-diluted semen varied. We would almost certainly not have ascribed an inhibitory effect of glutamate on respiration on the basis of data given in Table IV. These data do, however, not vitiate the conclusions drawn from Tables II and V. They indicate that the extent of the response may vary.

What factors are involved in this variation we do not know for to the best of our knowledge identical laboratory methods were used. We have no evidence that ageing of the roosters or that season of collection of the semen affects the response. It must be emphasized that our conclusions are based on incubation periods of 2-3 hours. As hourly respiration rate decreases more sharply after dilution with Tyrode diluent than with the other diluents, this restriction must be kept in mind in comparisons of diluents.

We believe that the conclusion that a higher respiration rate is obtained with a 50:

TABLE V. Effect of Glutamate and Glycine on Metabolic Rates of Fowl Semen.

Diluent	Respiration, $\mu\text{l}/10^8/3 \text{ hr}$	Fructose used, $\mu\text{g}/10^8/3 \text{ hr}$		Amino acid used, $\mu\text{g}/10^8/3 \text{ hr}$	N
		Aerobic	Anaerobic		
Tyrode	11.71	241.22	161.70	—	4
Glutamate	6.25*	14.88*	30.92*	6.1	4
Tyrode	13.14	—	—	—	9
Tyrode (84p) + 1/3 M glycine (16p)	7.87*	—	—	.66	9

* Different from treatment without amino acid ($P < .01$).

50 ratio of phosphate buffer and glutamate is not jeopardized by the somewhat lower than "normal" rate of respiration obtained with 100% phosphate, since experimental results are based on randomized complete block designs and the higher respiration rate was observed in 5 out of 7 cases. The nature of this synergistic effect of glutamate and phosphate is not clear, mainly because so little is known of the metabolism of fowl sperm.

The sharper decrease in respiration rate after dilution of fowl semen with Tyrode diluent may be related to the distinctive action of the chloride ions on sperm(1). Koefoed-Johnsen and Mann(9), observed that surfactants caused inhibition of fructolysis, respiration and motility. The chloride ion probably does not cause immediate disruption of the normal sperm morphology but eventually may act as surfactants do. Whether sperm stored in Tyrode diluent respond with a higher respiration rate to succinate than do phosphate diluted stored sperm should be investigated. Such a phenomenon was observed by Koefoed-Johnsen and Mann(9) after treatment of mammalian semen with surfactants.

The inhibition of respiratory activity by glutamate is, however, not attributable to replacement of chloride, for glutamate also inhibited respiration rate when compared to a phosphate diluent. Other data from our laboratory did not give evidence that phosphate causes damage to fowl sperm(1).

Glycine, which is known to act as a chelating agent(9), and glutamate may have a si-

milar effect on respiratory activity partly because of the shared characteristic of acting as a chelating agent. In view of the fact that tetracycline hydrochloride was added to all diluents and in view of its chelating effect (10) it seems that further investigation is necessary.

Summary. Glutamate, which can be catabolized by fowl semen, has an inhibitory effect on respiratory rate and in some experiments on rate of fructolysis. This effect may be explained partly by the catabolism of the glutamate and partly by its chelating effect. Glycine, which acts similarly to glutamate, is not catabolized by fowl semen.

We want to express our gratitude to Dr. M. C. Nesheim for critically reading the manuscript and for valuable suggestions.

1. El Zayat, S., van Tienhoven, A., *Am. J. Physiol.*, 1961, in press.
2. Snedecor, G. W., *Statistical Methods* 5th Ed., Iowa Univ. Press, Ames, 1956.
3. Wilcox, F. H., Shorb, M. S., *Am. J. Vet. Res.*, 1958, v19, 945.
4. van Tienhoven, A., *J. Agric. Sci.*, 1960, 54, 67.
5. Tyler, A., Rothschild, Lord, *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v76, 52.
6. Flipse, R. J., *Science*, 1956, v124, 228.
7. Flipse, R. J., Benson, A. A., *Exp. Cell. Res.*, 1957, v13, 611.
8. Koefoed-Johnsen, H. H., Mann, T., *Biochem. J.*, 1954, v57, 406.
9. Lorenz, F. W., Tyler, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1951, v78, 57.
10. Weinberg, E. D., *Bact. Rev.*, 1957, v21, 46.

Received February 3, 1961 P.S.E.B.M., 1961, v106.

Effect of Hypophysectomy on Adrenocortical Response to Bilateral Carotid Constriction. (26482)

EDWARD G. BIGLIERI AND WILLIAM F. GANONG

Metabolic Unit for Research in Arthritis and Allied Diseases and Department of Physiology, University of California School of Medicine, San Francisco

Gann, Mills and Bartter(1) have demonstrated that bilateral constriction of the common carotid artery causes an increase in aldosterone secretion in normal dogs. Considerable evidence has accumulated that

ACTH affects aldosterone secretion(2,3). This fact makes it difficult to decide whether increased aldosterone secretion in any given experiment is due to increased secretion of ACTH or to a proposed nonpituitary aldosterone

terone-stimulating hormone(4), when the experiment has been performed in animals with intact pituitaries. The present experiments were done in order to determine whether the response to carotid constriction is dependent on increased secretion of ACTH. Some of the results have been reported previously in abstract form(5).

Materials and methods. The right lumbodorsal veins of 11 male mongrel dogs weighing 12.0-15.8 kg were cannulated by the method of Hume and Nelson(6) under pentobarbital anesthesia. Transbuccal hypophysectomy was then performed in 7 of the dogs. Fifty-five minutes after operation 2 hypophysectomized dogs were given 5 milliunits (mU) ACTH (Upjohn) intravenously, followed by a constant infusion of 30 mU of ACTH per hour. This amount of ACTH was found in previous experiments to maintain 17-hydroxycorticoid output at 50-75% of maximum(7). Ninety-five minutes after hypophysectomy, adrenal cannulation, or both, the common carotid arteries were constricted in each of the 11 dogs by tying a ligature around the artery and an 18-gauge hypodermic needle, after which the needle was withdrawn.

Adrenal venous blood specimens were collected from the 2 dogs receiving ACTH before the constant infusion was started, and from all dogs 60 and 90 minutes after hypophysectomy and/or cannulation. Specimens were collected from 2 hypophysectomized dogs 15 minutes after carotid constriction, from the remaining 9 dogs 30 minutes after constriction, and from all 11 dogs 60 minutes after constriction. The adrenal venous blood specimens were centrifuged promptly and the plasma frozen for subsequent analysis of aldosterone content by the double isotope derivative technic of Kliman and Peterson(8), and 17-hydroxycorticoids by the Silber-Porter method(9). Femoral and lingual blood pressures were monitored throughout the experiment with Satham strain gauges and a Grass Model 4 polygraph. Gross examination of the sella and hypothalamus at autopsy showed all hypophysectomies to be complete.

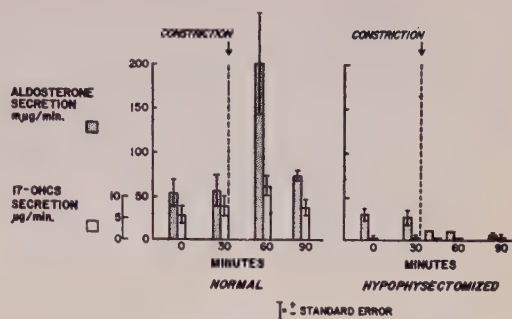


FIG. 1. Effect of carotid artery constriction on adrenal secretion in dogs.

Results. Mean aldosterone outputs 60 and 90 minutes after cannulation in the nonhypophysectomized dogs were 56 ± 18 (mean \pm standard error of the mean) and 57 ± 22 $\mu\text{g}/\text{minute}$, with a rise to 202 ± 75 $\mu\text{g}/\text{minute}$ 30 minutes after carotid constriction, and to 73 ± 8 $\mu\text{g}/\text{minute}$ 60 minutes after constriction. However, the magnitude of the rise varied considerably in individual animals, and the mean value 30 minutes after constriction is not significantly different from the pre-constriction control. Mean control 17-hydroxycorticoid output values in these stressed dogs were 5.7 ± 2.6 and 7.8 ± 3.5 $\mu\text{g}/\text{minute}$, rising to 12.3 ± 3.1 and 7.4 ± 2.5 $\mu\text{g}/\text{minute}$ 30 and 60 minutes after carotid constriction (Fig. 1). In the 5 untreated hypophysectomized dogs, control aldosterone output values were 30 ± 7.8 and 26 ± 8.5 $\mu\text{g}/\text{minute}$. Fifteen minutes after carotid constriction, the mean output (2 dogs) was 11 $\mu\text{g}/\text{minute}$. Thirty minutes after constriction, it was 10 ± 2.1 (3 dogs), and 60 minutes after constriction it was 6 ± 1.8 $\mu\text{g}/\text{minute}$. The corresponding values for 17-hydroxycorticoid output were 0.5 ± 0.7 and 0.7 and 0.9 $\mu\text{g}/\text{minute}$ in the control period and 0.9 , 0.7 and 0.6 ± 0.8 $\mu\text{g}/\text{minute}$ after constriction.

The aldosterone and 17-hydroxycorticoid output values for the 2 hypophysectomized dogs which were treated with ACTH are shown in Table I. Seventeen-hydroxycorticoid output was increased during the infusion, but no consistent change occurred in aldosterone or 17-hydroxycorticoid output after carotid constriction.

Tying the carotid artery around an 18-

TABLE I. Effect of Carotid Constriction on Adrenocortical Secretion in Hypophysectomized Dogs Infused with ACTH.

	Control	30 mU ACTH/hr				
	45	Time after hypophysectomy (min.)				
		60	90	95	125	155
Aldosterone (m μ g/min.)						
Dog 1	27	30	80	Carotid con- striction	50	80
" 2	60	80	82		25	57
17 OHCS (μ g/min.)						
Dog 1	.2	4.2	4.8		3.8	4.0
" 2	.2	5.3	4.5		5.2	7.2

gauge needle caused a 30-60 mm Hg fall in lingual arterial pressure, and a decrease in lingual pulse pressure from a mean of 43 to a mean of 5 mm Hg. The systemic pressure rose sharply after constriction, then fell gradually to its precontraction level in 1 hour.

Discussion. The present data show clearly that although a rise in aldosterone secretion may follow bilateral constriction of the common carotid arteries in the normal dog, it does not occur in the hypophysectomized animal. It therefore appears that carotid constriction leads to increased secretion of ACTH, which is responsible for any increase in aldosterone secretion. The rise in 17-hydroxycorticoid secretion that occurred in the normal dogs after carotid constriction supports this conclusion.

It is possible that hypophysectomy abolishes the responsiveness of the adrenal to a nonpituitary aldosterone-stimulating factor. Since the administration of ACTH causes prompt rises in the secretion of both 17-hydroxycorticoids and aldosterone(10) up to 6 hours after hypophysectomy, loss of responsiveness probably does not play a role in these studies. To rule out the possibility that some circulating ACTH is necessary for adrenal responsiveness, 2 hypophysectomized dogs were given ACTH by constant infusion while the adrenocortical response to carotid constriction was measured. The dose of ACTH chosen produced and maintained a 4-fold rise in 17-hydroxycorticoid secretion, but subsequent carotid constriction produced no change in the secretion of these hormones or aldosterone. Thus increased aldosterone secretion observed in nor-

mal dogs seems to be due to increased secretion of a pituitary factor, and this factor is probably ACTH.

Summary. To determine whether the increase in aldosterone secretion following constriction of the carotid arteries was due to release of pituitary ACTH, 4 normal dogs, 5 acutely hypophysectomized dogs and 2 acutely hypophysectomized dogs given ACTH by constant infusion were subjected to constriction of both common carotid arteries. A variable rise in aldosterone secretion occurred in dogs with intact pituitaries after carotid constriction, with an associated increase in 17-hydroxycorticoid secretion. In hypophysectomized dogs and in ACTH-infused hypophysectomized dogs, this rise failed to occur. The data support the conclusion that the increase in aldosterone secretion following carotid constriction is due to pituitary release of ACTH.

The authors wish to express their thanks to Miss Angela Boryczka, Mr. Roy Shackleford and Miss Bobbye Duren for technical assistance. ACTH used in these studies was generously supplied by The Upjohn Co. This work was supported by a grant-in-aid from the Commonwealth Fund and a grant from Nat. Inst. for Neurol. Dis. and Blindness.

1. Gann, D. S., Mills, I. H., Bartter, F. C., *Fed. Proc.*, 1960, v19, 605.

2. Ganong, W. F., Lieberman, A. H., Daily, W. J. R., Yuen, V. S., Mulrow, P. J., Luetscher, J. A., Jr., Bailey, R. E., *Endocrinology*, 1959, v65, 18.

3. Ganong, W. F., Forsham, P. H., *Ann. Rev. Physiol.*, 1960, v22, 579.

4. Farrell, G., *Physiol. Rev.*, 1958, v38, 709.

5. Biglieri, E. G., Ganong, W. F., *The Physiologist*, 1960, v3, 21 (abstract).

6. Hume, D. M., Nelson, B., *Surgical Forum, Am. Coll. of Surgeons*, 1954, W. B. Saunders Co., 1955, p5.

7. Ganong, W. F., unpublished observations.

8. Kliman, B., Peterson, R. E., *J. Biol. Chem.*, 1960, v235, 1639.

9. Silber, R. H., Porter, C. C., *ibid.*, 1954, v210, 923.

10. Mulrow, P. J., Ganong, W. F., *Fed. Proc.*, 1960, v19, 152 (abst).

Received February 2, 1961. P.S.E.B.M., 1961, v106.

Bile Duct Obstruction and Regeneration of the Liver.* (26483)

CARL H. ANDRUS,[†] GISELLE PECHET AND RICHARD A. MACDONALD

*Department of Pathology, Harvard Medical School and the Mallory Institute of Pathology,
Boston City Hospital*

It was recently observed in this laboratory that ligation of the common bile duct of the rat stimulated proliferation of liver cells in the absence of liver cell necrosis(1). The present work was carried out to study further the effect of biliary obstruction on hepatic regeneration, and to compare regeneration due to biliary obstruction with that following partial hepatectomy. Incorporation of tritiated thymidine (H^3 -thymidine) into deoxyribonucleic acid (DNA) was quantitated in autoradiographs, and mitoses were counted in histologic sections to detect and quantitate regeneration(2).

Higgins and Anderson(3) performed partial hepatectomies after bile duct ligation in rats: Wet and dry weights were correlated with the histologic appearance and with hepatic mitoses; it was found that weight was restored as well or better than in normal rats subjected to partial hepatectomy. On the basis of gross and histologic observations, however, they interpreted restoration of weight in obstructed liver to be due to engorgement with bile and blood, and they concluded that regeneration was impaired after bile duct ligation. Cameron(4) studied survival of slices of normal and obstructed liver placed as autotransplants in the omentum of rats. He found that in liver from animals with bile duct obstruction liver cells did not

survive as long as from normal rats. He concluded that bile duct ligation inhibited regeneration. Ferguson, Rogers and Vars(5) studied total liver weight, protein content, and mitotic activity and found that in rats that had undergone bile duct ligation at the time of partial hepatectomy, regeneration was as great or greater than regeneration in partially hepatectomized normal liver. Weinbren(6) studied rat liver subjected to partial hepatectomy after bile duct ligation. He compared gain in weight of remaining liver lobes, and found no difference between obstructed and normal liver; he concluded that bile duct ligation did not inhibit regeneration.

Materials and methods. Forty male rats of the Sprague-Dawley strain,[‡] separated by litter-mates into 24-hour and 48-hour experimental groups were used. Animals were obtained at 21 days of age, and were maintained in this laboratory until 119 to 124 days of age, at which time they weighed 315 to 415 g. They were housed individually in an air-conditioned room, and were fed laboratory chow and tap-water *ad libitum*. Animals were divided into groups as follows: 12 underwent ligation of the common bile duct midway between liver and duodenum, followed immediately by partial hepatectomy of approximately 50% of the liver(7). Twelve rats were subjected to partial hepatectomy alone; 8 to ligation of the bile ducts alone, and 8 to a sham operation consisting of

*Aided by grants from U.S.P.H.S. and in part by Contract from Office of the Surgeon General U. S. Army.

[†]Third year student, University of Rochester School of Medicine, Rochester, N. Y.

[‡]Purchased from Charles River Breeding Laboratories, Boston, Mass.

TABLE I. Uptake of Tritiated Thymidine and Mitotic Activity of Rat Liver Cells Following Ligation of Common Bile Duct and Partial Hepatectomy.

Operative procedure	Hr after operation	No. rats	Tritium labeled hepatic nuclei*	Mitoses*
Partial hepatectomy	24	6	24,688 \pm 9,669†	695 \pm 409†
Bile duct ligation & partial hepatectomy	24	6	3,151 \pm 859	54 \pm 66
Bile duct ligation	24	4	22 \pm 10	4 \pm 19
Sham operation	24	4	27 \pm 9	0
Partial hepatectomy	48	6	3,761 \pm 1,331	288 \pm 180
Bile duct ligation & partial hepatectomy	48	6	8,863 \pm 3,380	698 \pm 254
Bile duct ligation	48	4	4,958 \pm 3,362	298 \pm 134
Sham operation	48	4	8 \pm 3	0

* Expressed per 100,000 hepatic cell nuclei.

† \pm stand. dev.

laparotomy and manipulation of the liver and bile ducts.

Twenty of the rats were sacrificed at 24 hours, and the remaining twenty 48 hours after operation as shown in Table I. Four hours prior to sacrifice H³-thymidine[§] was administered under ether anesthesia *via* one femoral vein, in a dose of one-half microcurie per gram body weight. Animals were sacrificed by etherization and decapitation. Slices of liver were fixed in 10% neutral buffered formalin. Histologic sections were stained with hematoxylin and eosin and stripping film autoradiographs were prepared as described elsewhere(2). Kodak AR-10 film was used, with an exposure time of 4 weeks. In autoradiographs, tritium labeled hepatic nuclei were counted in 100 consecutive 400 \times fields containing parenchymal cells; in histologic sections mitotic figures in hepatic cells were counted in 50 consecutive 400 \times fields. Results were expressed per 100,000 hepatic nuclei.

An attempt was made to carry out similar studies 3 weeks after bile duct obstruction and partial hepatectomy, but because of a high mortality of rats with ligation and division of bile ducts, apparently due to liver failure and to infection, this part of the experiment was discontinued.

Results. All operated rats lost 10 to 30 g during the one or the 2 days between opera-

tion and sacrifice. Rats which had undergone partial hepatectomy had fibrinous adhesions of intestine, stomach or omentum to the stumps of resected lobes. In rats with bile duct ligation and partial hepatectomy the livers were bile stained in addition. Bile ducts in animals with ligation showed little or no dilatation 24 hours later; 48 hours after operation the ducts proximal to the ligation were dilated and contained an estimated 0.5 ml of bile. Sham operated rats showed no intraperitoneal changes.

Histology. The livers of sham operated rats were normal; in animals that had undergone partial hepatectomy, liver cells were enlarged, with finely vacuolated cytoplasm(7). In rats with bile duct ligation alone the changes were similar to those described by Cameron and Oakley(8). At 24 hours and more markedly at 48 hours bile ducts were increased in number in portal areas; cells of bile duct epithelium were enlarged and contained large, pale nuclei. In portal areas connective tissue appeared edematous, with more fibroblasts than usual, and there were increased numbers of lymphocytes and polymorphonuclear leukocytes. Liver cell nuclei were enlarged and pale staining. As noted previously(1) necrosis was not a prominent feature of these livers; there were scattered degenerated liver cells with pyknotic nuclei, occasional "hyaline bodies" composed of cytoplasm of degenerated liver cells, and in 2 livers there were rare "Gombault infarcts." (8). The histologic findings were similar in

§ Schwarz Laboratories, Mount Vernon, N. Y., specific activity 1.9 curies per millimole.

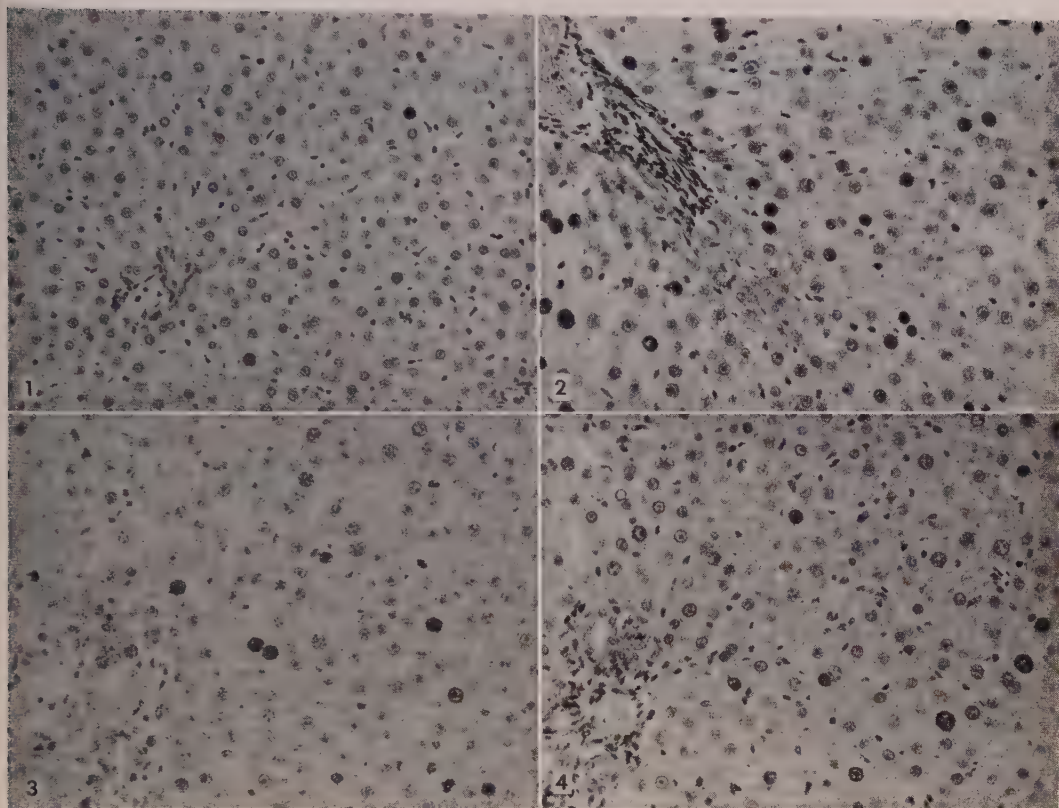


FIG. 1. Autoradiograph of liver of normal rat, inj. with H^3 -thymidine 4 hr prior to sacrifice. One labeled liver cell nucleus is present in upper right portion. Hematoxylin $\times 200$.

FIG. 2. Autoradiograph of liver of rat subjected to partial hepatectomy and inj. with H^3 -thymidine 24 hr later. Approximately 40 labeled liver cell nuclei. Hematoxylin $\times 200$.

FIG. 3. Autoradiograph of liver of rat subjected to ligation of common bile duct and partial hepatectomy, and inj. with H^3 -thymidine 24 hr later. Approximately 20 labeled liver cell nuclei. Hematoxylin $\times 200$.

FIG. 4. Autoradiograph of liver of rat subjected to ligation of common bile duct and inj. with H^3 -thymidine 48 hr later. Approximately 20 labeled liver cell nuclei. Hematoxylin $\times 200$.

rats that had undergone both bile duct ligation and partial hepatectomy.

Autoradiographs and Mitotic Counts.

Twenty-four hours after operation, rats that had undergone partial hepatectomy and common bile duct ligation showed significantly less H^3 -thymidine uptake and mitotic activity of hepatic cells ($P < 0.01$) than rats subjected to partial hepatectomy alone. (Table I) H^3 -thymidine uptake and mitoses in livers of rats with ligation of the common bile duct were not significantly different from normal.

To confirm the findings at 24 hours, this portion of the experiment was repeated with an additional 17 rats; the results were essentially the same.

At 48 hours after operation, rats with par-

tial hepatectomy and bile duct ligation showed significantly more H^3 -thymidine uptake and mitoses ($P < 0.05$) than rats with partial hepatectomy alone. Rats with bile duct ligation alone showed markedly increased H^3 -thymidine uptake and mitotic activity of liver cells, comparable to the 48 hour values of rats that had undergone partial hepatectomy.

H^3 -thymidine uptake by bile duct epithelium was increased beginning 24 hours after operation in rats with bile duct ligation and in those with combined bile duct ligation and partial hepatectomy.

Comment. This work suggests that liver cell proliferation occurring as a result of bile duct obstruction may be due to sublethal,

histologically inapparent damage to liver cells. The regenerative response on the part of liver cells begins 40 to 48 hours after common duct obstruction; in time of onset, it is thus different from the regeneration that occurs after partial hepatectomy, which is advanced at 24 hours. Further, there is an early, brief interference of biliary obstruction with regeneration after partial hepatectomy, and there is a long interval of 48 hours between obstruction of the bile ducts and appearance of increased DNA synthesis and mitoses in liver cells, during which time injury, and response to injury, may occur. Substances such as serotonin(9) and thio-urea(10) have been shown to cause increased liver cell mitoses in the absence of necrosis, probably due to sublethal damage to cells which is followed by proliferation.

Summary. DNA synthesis and mitosis in liver cells are partially inhibited 24 hours after operation in rats subjected to both partial hepatectomy and bile duct obstruction; at 48 hours regeneration is greater than after partial hepatectomy alone, although not as marked as that seen at 24 hours after partial

hepatectomy. Liver cell proliferation occurs 40 to 48 hours after obstruction of the common bile duct, and is presumably the result of histologically inapparent injury to liver cells.

1. MacDonald, R. A., Pechet, G., *A.M.A. Arch. Path.*, in press.
2. MacDonald, R. A., Mallory, G. K., *Lab. Invest.*, 1959, v8, 1547.
3. Higgins, G. M., Anderson, R. M., *A.M.A. Arch. Path.*, 1932, v14, 42.
4. Cameron, G. R., *J. Path. and Bact.*, 1935, v41, 283.
5. Ferguson, C. C., Rogers, C. S., Vars, H. M., *Am. J. Physiol.*, 1949, v159, 343.
6. Weinbren, K., *Brit. J. Exp. Path.*, 1953, v34, 280.
7. Higgins, G. M., Anderson, R. M., *A.M.A. Arch. Path.*, 1931, v12, 186.
8. Cameron, G. R., Oakley, C. L., *J. Path. and Bact.*, 1932, v35, 769.
9. MacDonald, R. A., Schmid, R., Hakala, T. R., Mallory, G. K., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v101, 83.
10. Rachmilewitz, M., Rosin, A., Doljanski, L., *Am. J. Path.*, 1950, v26, 937.

Received February 3, 1961. P.S.E.B.M., 1961, v106.

Lens Sodium and Potassium Concentrations During Galactose Induced Cataractogenesis.* (26484)

F. W. HEGGENESS AND J. HILL (Introduced by E. S. Nasset)

Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The electrolyte composition of the ocular lens of the rat is greatly modified during formation of galactose-induced cataracts. Of particular interest is a reduction in potassium and elevations in sodium and calcium concentration(1,2). The relationship between the changes in these cations and lens opacification is not known. Certain biochemical studies suggest that cataractogenesis is the consequence of an inhibition of lens metabolism by galactose(3,4). It has also been demonstrated that maintenance of the lens con-

tent of sodium and potassium is energy dependent(5) and the changes observed in these cations during cataractogenesis may reflect the effect of galactose on lenticular metabolism. The proposal has been made that the concentration of these cations in the lens during cataractogenesis could serve as an index to the progression of the lesion(6).

The present studies were made to determine the time course of the modification of lens sodium and potassium in relation to development of galactose-induced lens opacification. Large differences are known to exist in susceptibility of several strains of albino rats to the cataractogenic property of galac-

* This work was supported in part by a grant from Nat. Inst. Health.

tose(7,8). The degree to which susceptibility to cataractogenesis influences the magnitude and time course of the cation changes was studied by using 3 strains of albino animals known to differ markedly in this regard (8). Animals of the Carworth Farm Nelson strain fed a 60% galactose diet develop cataracts after 32 days. Total time to lens opacification in weanling rats of Rochester ex Wistar and Holtzman strain animals is 20 and 13 days respectively.

Method. Weanling male rats of Carworth Farm Nelson (CFN), Rochester ex Wistar (RW) and Holtzman (H) strains were housed in a room maintained at constant temperature. Food and water were available *ad libitum*. The diet contained 60% carbohydrate, 21% casein, 15% fat (Crisco), 4% salt mixture plus a complete vitamin supplement. Control diets contained 60% glucose; experimental diets contained 30% glucose plus 30% galactose or 60% galactose. Except when specifically indicated, experimental animals were fed the 60% galactose diet. Animals were sacrificed by decapitation, lenses were removed intact, blotted, weighed and ashed at 500°C for 24 hours. The ash was dissolved in dilute HCl and potassium and sodium determined by flame photometry. Lens calcium was precipitated as oxalate and determined by a modification of the method of Diehl and Ellingboe(9). Concentrations of these cations are reported as mEq/Kg tissue.

Results. Nutritional State of Animals. Galactose fed animals had polyuria and polydipsia with weight gains significantly below that of glucose fed animals. There was no difference in rate of weight gain of galactose-fed animals of the strain most resistant (CFN) or susceptible (H) to cataractogenesis (Table I).

In the experimental animals of all strains studied, equatorial vacuolization, characteristic of the initial stage of cataract development, appeared between days 6 and 10. During the next 3 to 4 days, vacuoles increased in size and the extent of lens involved, then remained relatively unchanged until 24 to 48 hours prior to opacification. At this time

TABLE I. Body Weights of Carworth Farm Nelson (CFN) and Holtzman (H) Strain Rats Fed a 60% Galactose Diet.

Day	CFN strain	Holtzman strain
0	45.0 \pm 1.4*	44.0 \pm 1.3
3	50.3 \pm 1.0	44.4 \pm 2.8
6	58.5 \pm .5	62.0 \pm 3.0
9	68.4 \pm 1.0	68.9 \pm 3.7
12	76.7 \pm 1.5	78.4 \pm 3.9
15	87.0 \pm 3.2	87.1 \pm 4.4
18	100.7 \pm 3.7	99.0 \pm 2.4

* Mean \pm S.E. of 9 animals.

rapid progression to complete opacification occurred.

Lens Ca Concentrations. Lens calcium concentrations were determined in control and experimental animals of the CFN strain only. No differences in calcium content between these groups developed during the first 20 days (Fig. 1). A slight increase in calcium content may develop during days 20 to 30. Other studies have indicated that the lens Ca concentration of 7 week old control animals of this strain is 1.5 ± 0.2 mEq/Kg.

Lens K. Concentration. Earlier studies have shown that in the CFN strain of rat, lens potassium on the second day of galactose feeding is not different from that of the controls (37.6 ± 3.6 mEq/Kg and 43.6 ± 2.8 mEq/Kg). The later time course of the lens potassium concentration in control and experimental animals of this strain is shown in Fig. 1. Lens potassium concentration is significantly below control values on day 4; this decreased concentration is maintained approximately 48 hours when concentration increases to a value approaching that found in control animals. Concentration of this cation is maintained at approximately 75% of control values from day 8 until day 16. At this time, 12 days before opacification, another precipitous drop occurs to a value comparable to that of the mature cataracts.

In another group of animals of the CNF strain fed the 30% galactose diet, initial opthalmologic changes were observed on day 14 and after 60 days only 50% of the animals had developed complete cataracts. On the twentieth day of feeding the 30% galactose diet, lens potassium concentration was 13.6 ± 6.1 mEq/Kg and at 60 days, in the lenses

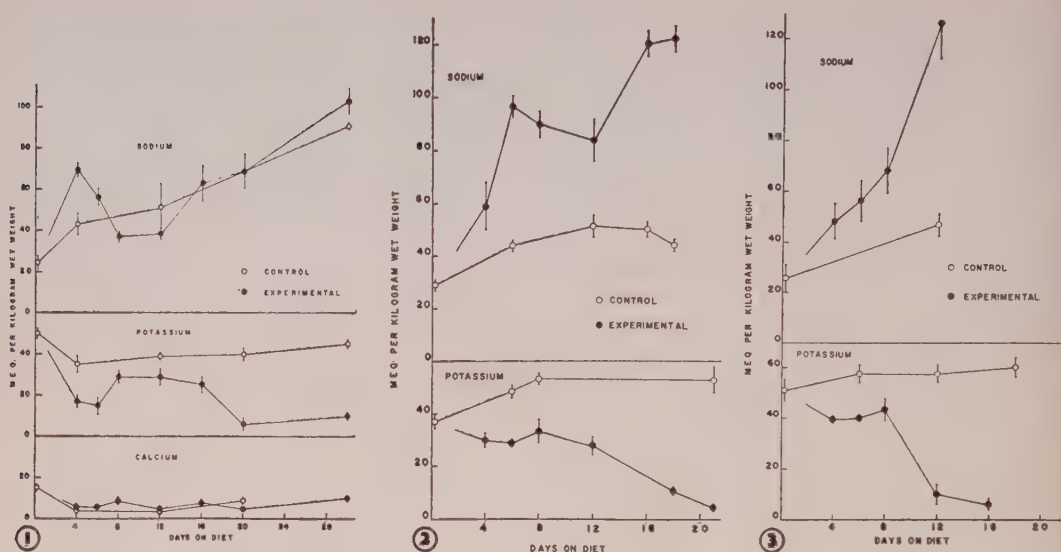


FIG. 1. Lens sodium potassium and calcium concentrations in weanling Carworth Farm Nelson strain rats fed diets containing 60% glucose or 60% galactose.

FIG. 2. Lens sodium and potassium concentrations in Rochester ex Wistar strain weanling rats fed diets containing 60% glucose or 60% galactose.

FIG. 3. Lens sodium and potassium concentrations in Holtzman strain weanling rats fed diets containing 60% glucose or 60% galactose.

that had not opacified, 23.1 ± 1.4 mEq/kg. Lens potassium concentration in glucose fed animals on day 20 and 50 was 40.0 ± 1.5 and 51.2 ± 5.0 mEq/Kg respectively.

In the RW and H strains a initial reduction, similar to that observed in CFN animals, occurred in potassium concentration on day 4 (Figs. 2 & 3). In these strains, no subsequent partial recovery occurred. Concentration of this cation remained at approximately 60% of control value until day 8 in the most susceptible strain (H) and day 12 in the RW strain; then, at 5 and 8 days, respectively, prior to opacification, potassium concentration decreased to values characteristic of mature cataracts.

Lens Sodium Concentration. In galactose fed animals of the CFN strain, lens sodium concentration followed a pattern generally reciprocal to that of potassium (Fig. 1). A sharp initial rise was followed by a temporary restitution of control values. The time course of these changes in sodium was the same as that described for potassium.

In the RW strain of rats (Fig. 2) sodium concentration rose initially to value 200% of that found in control animals. No partial reversal occurs in this more susceptible

strain and this concentration was maintained until days 12 to 16 when a further increase occurred. As with the CFN strain, changes in sodium concentration were reciprocal to, but followed the same time course as, those found for potassium.

In H strain, changes in lens sodium changes paralleled those seen in RW strain animals (Fig. 3).

Discussion. The polyuria and polydipsia of the galactose-fed animals reflect the inefficiency of utilization of this sugar. The finding of equal weight gains in animals resistant (CFN) and susceptible (H) to cataractogenesis suggests that differences in capacity to metabolize galactose are not the basis for strain differences in cataract susceptibility. Other studies(7) have indicated that the strain differences, in susceptibility to xylose-induced cataractogenesis, appear to reside in the lens itself.

In galactose fed animals of all of the 3 strains, modification of concentrations of potassium and sodium is evident on day 4, prior to appearance of initial ophthalmologic changes. In only the most resistant strain (CFN) was there a partial recovery toward control values. In the 2 more susceptible strains of

rats, lens sodium concentration remains relatively unchanged until the time that potassium concentration decreased, at which time sodium concentration rose again. The finding that sodium and potassium change reciprocally in the CFN strain suggests that this partial recovery reflects a real difference between this relatively resistant and the other more susceptible strains. Length of time that potassium concentrations are maintained at 60 to 70% of control values varies directly with degree of strain resistance. In all strains a precipitous drop in lens potassium occurred prior to opacification. Again, length of time between the second fall in potassium concentration and lens opacification varies directly with strain resistance.

These findings suggest that a critical period in studies of the resistance of the CFN strain might be centered around days 6 to 8. A new energy-yielding metabolic pathway or an augmentation of those already present may permit partial recovery to the control concentrations of sodium and potassium ions. In this strain, opacification does not occur, in the presence of a depressed lens potassium concentration for 12 days while ingesting a 60% galactose diet and for 25 days when fed a 30% galactose diet.

A single sodium or potassium analysis of the lens does not provide an adequate index of the progression of cataract formation or even to the proximity of opacification. The time course and magnitude of the changes in lens sodium and potassium concentration appear to be characteristic of and dependent upon the relative susceptibility of the strain employed.

Summary. The lens concentrations of sodium and potassium were determined during galactose-induced cataractogenesis in 3 strains of albino rats. Four days after galactose feeding was initiated, lens potassium concentration decreased to 60% and sodium concentration increased to 175% of control values. In resistant, but not in susceptible, strains of animals partial return to control concentrations of both of these ions occurred. The changed lens sodium and potassium concentrations were maintained for 4-16 days depending on degree of resistance in each strain. Lens potassium then decreased and sodium concentration increased prior to actual opacification. The comparable weight gains of *ad libitum* fed animals of resistant and susceptible strains suggested that resistance or susceptibility to cataractogenesis resides in lens itself.

The authors are grateful for the technical assistance of Kathleen Srokose.

1. Salit, P. W., Swan, K. C., Paul, W. D., *Am. J. Ophthalm.*, 1942, v25, 1482.
2. Hill, J. C., Heggeness, F. W., *Am. J. Physiol.*, 1959, v197, 85.
3. Schwarz, V., Goldberg, L., *Biochem. et biophys. acta*, 1958, v30, 298.
4. Lerman, S., *Science*, 1959, v130, 1473.
5. Harris, J. C., Gruber, L., Talman, E., Hoskinson, G., *Am. J. Ophthalm.*, 1959, v48, 528.
6. Sbordone, G., *Archivio di Ottalmologia*, 1958, v62, 73.
7. Mitchell, H. S., *J. Nutrition*, 1936, v12, 447.
8. Heggeness, F. W., *Am. J. Physiol.*, 1960, v198, 625.
9. Diehl, H., Ellingboe, J. L., *Anal. Chem.*, 1956, v28, 882.

Received February 6, 1961. P.S.E.B.M., 1961, v106.

Tissue Catecholamine Content of Cold-Acclimated Rats. (26485)

K. E. MOORE,* D. N. CALVERT,† AND T. M. BRODY (Introduced by M. H. Seevers)

Department of Pharmacology, University of Michigan Medical School, Ann Arbor

The role of the adrenal medulla in production of nonshivering thermogenesis was first reported by Cottle and Carlson(1). As a result of later work showing that norepinephrine (NE) had a very marked calorogenic action in cold-acclimated rats it was postulated that NE was the mediator of the nonshivering heat production in these rats(2).

Release of catecholamines in response to acute cold stress has been noted by several investigators(3). Catecholamine levels in the adrenal gland of rats during development of cold acclimation have been reported by Des Marais and Dugal(4), but the catecholamine content in other tissues of cold-adapted rats has not been previously studied. The effects of prolonged cold exposure on catecholamine content of rat brain, heart and adrenal are reported here.

Methods. Twenty-four female rats (Sprague-Dawley) were randomly divided into 2 groups. One group, mean weight 185 g (range 170-205 g), was placed in a cold room at 5-7°C. The other group, mean weight 174 g (range 165-195 g) remained in the animal colony at 25-27°C. After a period of 3-5 weeks, at which time a maximum metabolic response is obtained(5), the rats were sacrificed. The liver, kidney, heart and brain weights were recorded and the epinephrine (E) and NE content of the adrenal, heart and brain determined by a modification(6) of the fluorimetric method of Bertler *et al.* (7).

Results. At time of sacrifice both groups of rats appeared healthy. Those exposed to the cold had a mean weight of 215 g (range 205-235), those in the 25-27°C room, a mean weight of 198 g (range 180-220 g). Rates of increase in body weight for the 2 groups over the experimental period were not significantly

different. However, whether presented as absolute organ weights or fractional organ weights $\left(\frac{\text{organ weight}}{\text{body weight}} \times 100 \right)$, values of liver, kidney and heart of the cold-adapted animals were significantly greater than those of the control group (Table I). Brain weights of both groups were nearly identical. Since it has been shown(8) that brain weight is poorly correlated with body weight, fractional weights of brain are meaningless and therefore were not calculated.

Catecholamine content of the adrenal, brain and heart of normal and cold-adapted rats is recorded in Table II. Brain content of catecholamines is similar in both groups. Catecholamine content of the heart, when reported on a $\mu\text{g/g}$ wet weight basis, is markedly lower in the cold-adapted rats. This difference is apparently due to hypertrophy of the heart muscle during the period of cold exposure since if the catecholamine content per heart is calculated, the μg of NE are: normal, $0.57 \pm .12$ and cold, $0.54 \pm .16$ and for E are: normal, $0.07 \pm .01$ and cold, $0.05 \pm .03$. The differences between these values are not significantly different (for NE, $P = .6$; for E, $P = .4$). The content of both E and NE in the adrenal of cold-adapted rats was markedly increased over the values for control rats.

Discussion. In contrast to the reports of others(9), we found no significant decrease in rate of weight gain in cold exposed rats. Our results might be explained by the fact that female rats were used in this study while most previous studies were carried out on male rats. That this sex difference may account for our findings is strengthened by the report of Baker and Sellers(10), who found no obvious weight difference between female rats in cold and warm environments for 45 days. In agreement with Heroux and Gridgeman(8) we found a very obvious hypertrophy of the liver, heart and kidney but

* Present address: Dept. of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, N. H.

† Present address: Dept. of Pharmacology, Univ. of Kansas Medical Center, Kansas City, Kan.

TABLE I. Organ Weights of Normal and Cold-Adapted Rats.

Organ	N	Absolute weights*			Fractional weights†		
		Normal	Cold	P	Normal	Cold	P
Brain	11	1.74 ± .09	1.74 ± .04	—	—	—	—
Heart	11	.62 ± .08	.85 ± .06	<.01	.31 ± .03	.40 ± .03	<.01
Liver	10	6.3 ± .8	8.9 ± .6	<.01	3.2 ± .34	4.1 ± .24	<.01
Kidney	10	.79 ± .11	1.10 ± .09	<.01	.40 ± .04	.52 ± .06	<.01

* Mean and stand. dev. of organ weights in g.

† *Idem* expressed as % of body wt.

not the brain of cold-adapted rats.

The catecholamine content of the brains from normal and cold-exposed rats was essentially the same. Catecholamine content of the heart was reduced in the cold-adapted rat when expressed as $\mu\text{g/g}$ wet weight of the heart. This reduction however is apparently due to hypertrophy of the cardiac muscle. It would appear that the hypertrophy of the heart is not accompanied by a concomitant increase in the catecholamine content. A marked reduction of catecholamines by administration of reserpine produces changes in physiological responses of the heart, *e.g.* lowered heart rates(11). The slight reduction of catecholamines in the cold-adapted rats might in part be the cause of the reduced heart rates which have been reported in these animals(9).

In contrast to the reduction of catecholamines in adrenals of rats subjected to acute cold stress(13), it was found that upon chronic exposure to cold, adrenal catecholamine content was markedly increased. This confirms the work of Des Marais and Dugal (4), who, however, reported rather low initial values for E and NE so that their values for the cold-adapted animals were equivalent to normal values obtained by other workers. When calculated on the basis of μg amine/kg

body weight, normal values, 148 μg E and 36 μg NE, are comparable to those reported by Hokfelt for female rats(14). When calculated on the same basis, values for the cold-adapted adrenals were 174 μg E and 54 μg NE/kg body weight. Although the increase of NE is more marked than that of E, the content of both amines was significantly increased over normal values.

The increased content of adrenal catecholamines is not necessarily accompanied by increased secretion of these amines. To determine if the development of nonshivering thermogenesis of rats is dependent upon an increased output of catecholamines from tissue stores one must measure the actual output of amines from the tissues, perhaps by measuring urinary excretion of the amines and their degradation products. Why the catecholamines are so markedly increased in the adrenal and not in the heart or brain of cold-adapted rats is not clear. The increased ascorbic acid content of the adrenals in cold-adapted rats may play some role in this increase, either by protecting against destruction of formed catecholamines or by stimulating the synthesis of these amines(15).

Summary. Exposure of female rats to temperatures of 5-7°C for 3-5 weeks resulted in hypertrophy of the liver, heart and kidney.

TABLE II. Catecholamines in Cold-adapted and Normal Rats.

		Adrenal ($\mu\text{g}/\text{adrenal pair}$)		Brain ($\mu\text{g}/\text{g}$)		Heart ($\mu\text{g}/\text{g}$)	
Epinephrine	Normal	(11)	29.1 ± 4.7*	(11)	.04 ± .01	(11)	.11 ± .05
	Cold	(11)	37.4 ± 5.0	(10)	.04 ± .02	(11)	.06 ± .03
	P		<.01		≅.7		≅.02
Norepinephrine	Normal	(11)	7.0 ± 3.1	(11)	.45 ± .11	(11)	.94 ± .25
	Cold	(11)	11.6 ± 2.6	(10)	.47 ± .08	(11)	.64 ± .17
	P		<.01		≅.9		<.01

* Figures represent mean ± stand. dev. Numbers in parentheses represent No. of determinations.

In these rats the catecholamine content of the brain was unchanged, while that of the heart was reduced and the adrenal increased.

1. Cottle, W. H., Carlson, L. D., *Proc. Soc. Exp. Biol. and Med.*, 1956, v92, 845.
2. Hsieh, A. C. L., Carlson, L. D., Gray, G., *Am. J. Physiol.*, 1957, v190, 247.
3. Von Euler, U. S., *Fed. Proc.*, 1960, v19, suppl. 5, 79.
4. Des Marais, A., Dugal, L., *Can. J. Med. Sci.*, 1951, v29, 90.
5. Hannon, J. P., *Am. J. Physiol.*, 1958, v192, 253.
6. Moore, K. E., Brody, T. M., *J. Pharmacol.*, 1961, in press.
7. Bertler, A., Carlson, A., Rosengren, E., *Acta physiol. scand.*, 1958, v44, 273.
8. Heroux, O., Gridgeman, N. T., *Can. J. Biochem. Physiol.*, 1958, v36, 209.
9. Heroux, O., Campbell, J. S., *ibid.*, 1959, v37, 1263.
10. Baker, D. G., Sellers, E. A., *ibid.*, 1957, v35, 631.
11. Maling, H. M., Cohn, V. H., Jr., Highman, B., *J. Pharmacol.*, 1959, v127, 229.
12. Heroux, O., Depocas, F., Hart, J., *Can. J. Biochem., Physiol.*, 1959, v37, 473.
13. Allen, J. M., *J. Histochem., Cytochem.*, 1956, v4, 341.
14. Hokfelt, B., *Acta physiol. scand.*, 1951, v25, suppl. 92.
15. Hoijer, D. J., *Fed. Proc.*, 1960, v19, suppl. 5, 90.

Received February 13, 1961. P.S.E.B.M., 1961, v106.

Metabolism of Thyroxine in Hyperthyroidism in Cattle.*† (26486)

B. N. PREMACHANDRA† AND C. W. TURNER

Department of Dairy Husbandry, University of Missouri, Columbia

Mean biological half-life of thyroxine ($t_{1/2}$) in a group of 16 normal dairy cows was observed to be 2.47 days and a turnover rate of 28.4% per day(1). The present study was concerned with influence of hyperthyroidism on $t_{1/2}$ of thyroxine. Hyperthyroidism was defined in cattle in terms of 50 and 100% increase above their estimated winter thyroxine secretion rate (TSR).

Methods and materials. TSR was determined in late winter in lactating cows of several breeds by replacement technique(2). All animals were maintained in dry lot with free access to iodized salt. In each experiment 300 μ c of carrier-free l-thyroxine- I^{131} was injected intravenously and blood samples taken from jugular vein each day following injection of hormone. Measurements of radioactivity of plasma were made in a National Radiac scintillation well counter (Mod-

el SA-2D) and conventional corrections were made for decay of isotope and for background. Disappearance of radioactivity in plasma from day to day followed an exponential course. In all experiments 4 g Tapa-zole/1000 lb body weight/day was administered to each animal to prevent recycling of I^{131} from thyroxine- I^{131} metabolism(3). Plot of disappearance of I^{131} from plasma was extrapolated back to zero time (time of injection) and biological half-time ($t_{1/2}$) of thyroxine was determined graphically or from slope of regression line(1). $t_{1/2}$ represents time required by animal body to eliminate one-half of I^{131} in blood.

Eight animals, after they had attained peak milk production, were injected for several months with l-thyroxine (T_4) subcutaneously daily, 50% in excess of estimated winter TSR, and its effect on milk production noted. $t_{1/2}$ of l-thyroxine- I^{131} was determined during this period of hyperthyroidism. In 2 animals, (a Guernsey and Jersey-Holstein cross) the level of T_4 injection was further raised to equal twice normal estimated TSR of animal. $t_{1/2}$ of l-thyroxine- I^{131}

*Contribution from Missouri Agri. Exp. Station. Journal Series No. 2255. Approved by Director.

† Aided-in-part by a grant from U. S. Atomic Energy Commission.

‡ Present address: Inst. of Exp. Pathology, Jewish Hosp., St. Louis, Mo.

TABLE I. Influence of Hyperthyroidism on Biological Half-Life of Thyroxine- I^{131} in Dairy Cattle.

Animal No. and breed	Estimated normal daily TSR (mg/100 lb body wt)	Normal animals	Biological half-life ($T_{1/2}$) of thyroxine- I^{131}	
			Hyperthyroid	
			50% above normal	100% above normal
24 (Brown Swiss)	.5	2.33 \pm .05	1.07 \pm .02 ^{(+)*}	
25 (" ")	.4	2.25 \pm .06	.85 \pm .04 ⁽⁺⁾	
869 (Holstein)	.6	2.10 \pm .03	1.12 \pm .03 ⁽⁺⁾	
870 (")	.5	2.32 \pm .07	1.08 \pm .04 ⁽⁺⁾	
873 (")	.4	2.29 \pm .06	1.10 \pm .02 ⁽⁺⁾	
630 (Jersey)	.6	2.50 \pm .09	1.00 \pm .01 ⁽⁺⁾	
38 (Guernsey)	.4	2.35 \pm .07	1.10 \pm .04 ⁽⁺⁾	.88 \pm .21 ⁽⁻⁾
601 (Holstein-Jersey cross)	.5	2.40 \pm .06	1.05 \pm .03 ⁽⁺⁾	.89 \pm .25 ⁽⁻⁾
Mean	.49	2.32	1.05	

* Lactational response to thyroxine treatment was considered as positive ⁽⁺⁾ when an increase in milk production of at least 20% over pre-treatment level was obtained.

was again determined in these 2 animals. Effect on milk production was also noted.

Results. Half-life ($t_{1/2}$) of thyroxine- I^{131} for group of 8 normal cows was 2.32 days. When maintained in hyperthyroid state 50% above their estimated daily TSR by exogenous T_4 , the $t_{1/2}$ was reduced to 1.05 days, a reduction of 54.3% (Table I). Two cows injected with T_4 100% above TSR showed a further reduction ($t_{1/2} = 0.89$ days). The range of $t_{1/2}$ in individual cows was minimal.

All cows showed increases in milk yield of 20% or more at 50% above normal T_4 level, but showed little or no further increase at 100% above normal level.

Discussion. When dairy cows were maintained in a hyperthyroid condition by injection of T_4 50% above their winter TSR, it was observed that rate of disappearance of thyroxine- I^{131} from blood was doubled. Increasing T_4 injection to 100% above winter TSR further decreased $t_{1/2}$. Milk production in all cows at 50% above normal level of T_4 increased 20% or more and the increase was sustained for many weeks. Increase to 100%

above normal level of T_4 had little or no further influence upon lactation.

These data indicate that hyperthyroidism tends to increase rate of T_4 metabolism in somatic cells. While T_4 metabolism may occur by 3 pathways, (1) deiodination in cells and excretion of I^{131} in urine, (2) glyconjugation in liver and excretion in bile, and (3) oxidative deamination, present method determines only total elimination of thyroxine- I^{131} from blood.

From these observations it would appear that body has a mechanism for protection against (in cases of) hyperthyroidism by decrease in $t_{1/2}$ of thyroxine. In study of induced hyperthyroidism a dose of T_4 would not be directly related to degree of hyperthyroidism since $t_{1/2}$ of thyroxine would gradually decline as level of T_4 increased.

1. Pipes, G. W., Premachandra, B. N., Turner, C. W., *J. Dairy Sci.*, 1959, v42, 1606.
2. ———, *ibid.*, 1957, v40, 340.
3. Premachandra, B. N., Pipes, G. W., Turner, C. W., *J. Animal Sci.*, 1960, v19, 553.

Received February 13, 1961. P.S.E.B.M., 1961, v106.

Long-Term Effect of Estrogen and Progesterone on Mammary Gland Growth in 3-Methylcholanthrene Treated and Non-treated Ovariectomized Rats.* (26487)

HENRY C. DAMM† AND CHARLES W. TURNER
(With technical assistance of Mary E. Powell)

Department of Dairy Husbandry, University of Missouri, Columbia

Normal mammary gland growth and development of mammary gland carcinomas appear to be hormone dependent. Estradiol benzoate (EB) and progesterone (P), injected into rats in optimal levels, will produce mammary gland development (as determined by DNA estimation) equivalent to that produced during normal pregnancy(1). Further mammary gland growth may be obtained experimentally in the rat by concomitant administration of other hormones(2,3). Increased mammary gland growth has been observed after recurrent pregnancies in the mouse(4). Since above evidence indicated possibility of increased growth, it seemed desirable to determine effect of long term injection of optimal levels of EB and P on mammary gland growth in ovariectomized rat.

Rapidity of development of mammary gland carcinoma in rat by 3-methylcholanthrene has been shown to be dependent upon hormonal status of animal(5,6). Ovariectomized rats develop tumors much less rapidly or consistently than normal controls, while those treated with either estrogen or progesterone appear to develop carcinoma at a more rapid rate. Therefore, it was felt that effect of 3-methylcholanthrene on mammary gland growth produced by long term injection of optimal levels of EB plus P should be studied.

Materials and methods. Young, mature female albino rats of Sprague-Dawley-Rolfs-meyer strain were ovariectomized and fed a commercial laboratory animal feed. Two weeks after ovariectomy animals were di-

vided into groups receiving 1 μ g EB plus 3 mg P daily, 3-methylcholanthrene (3-me) alone, 3-me plus 1 μ g EB and 3 mg P daily, and 3-me plus 2 μ g EB plus 6 mg P daily. EB and P were dissolved in an olive oil carrier such that 0.1 ml solution was injected subcutaneously daily. Ten mg 3-me was administered orally by forced intubation twice weekly for a period of 7 weeks. This dose has been reported adequate for production of mammary gland carcinoma in approximately 50% of intact animals within this period(5). Groups of rats receiving EB plus P daily were sacrificed after 20, 30, 40 and 50 days. All groups receiving 3-me were sacrificed after 50 days. The 6 posterior mammary glands were removed and frozen. DNA was determined as described previously(1).

Results. Mean total DNA/100 g B.W. of rats receiving EB plus P for 20 days was significantly greater than ovariectomized rats receiving E alone (Table I). Although DNA values generally increased up to 40 days of injection, no significant differences were found between groups injected for varying lengths of time up to 50 days, indicating only slight additional growth is produced by long-term treatment with EB and P.

Mammary gland DNA of rats receiving 3-me alone was not significantly different from that of ovariectomized controls, indicating that this level of carcinogen was ineffective in production of mammary gland growth. DNA of mammary glands of rats receiving either 1 μ g EB plus 3 mg P daily for 50 days plus 3-me, or 2 μ g EB plus 6 mg P daily for 50 days plus 3-me was significantly depressed as compared to DNA of rats receiving only 1 μ g EB plus 3 mg P for 50 days, indicating an inhibitory mechanism of action of 3-me. No mammary tumor formation was observed in these rats on gross visual inspection.

* Contribution of Mo. Agr. Exp. Sta. Journal Series No. 2256. Approved by Director.

† Postdoctoral Research Fellow of Nat. Cancer Inst. This investigation supported in part by grants from U.S.P.H.S. and Am. Cancer Soc.

TABLE I. Effects of Estradiol Benzoate (EB), Progesterone (P) and 3-Methylcholanthrene on DNA of Rat Mammary Glands.

Treatment	No. of rats	Mean body wt, g	-6 posterior mammary glands-			
			DFFT,* mg	DNA (μ g)/DFFT (mg)	Total DNA, mg	DNA/100 g BW, mg
1 μ g EB + 3 mg P 20 days	15	221	430	37.5	16.13	7.3 \pm .60†
Idem 30 "	12	231	408	44.7	18.25	7.9 \pm .42
" 40 "	10	246	396	52.8	20.91	8.5 \pm .76
" 50 "	15	252	442	46.7	20.66	8.2 \pm .55
3-methyl cholanthrene, 50 days	12	270	243	36.6	8.91	3.3 \pm .40
3-methyl cholanthrene, 1 μ g EB + 3 mg P, 50 days	12	266	362	49.2	17.82	6.7 \pm .63
3-methyl cholanthrene, 2 μ g EB + 6 mg P, 50 days	12	272	375	44.96	16.86	6.2 \pm .52
Ovariectomized controls†	15	273	359	23.20		3.05 \pm .14
1 μ g EB, 19 days†	5	246	485	22.86		4.48 \pm .15
1 μ g EB + 3 mg P, 19 days†	19	269	590.1	35.65		7.72 \pm .25

* Dry, fat-free tissue.

† Data from Moon *et al.*(1).

‡ Stand. error of mean.

Discussion. Using DNA as an index of growth, it has been shown that a 1 to 3000 ratio of EB to P injected for 19 days produced growth equivalent to that observed in mammary glands of rats on 19th day of pregnancy(1). Recent evidence(7), however, indicated that approximately 40% of mammary gland growth occurs after parturition. Recurrent pregnancy has been shown to stimulate greater mammary gland growth in mouse(4), indicating that some form of hormonal stimulation over a longer period of time may be effective. Present study indicates that long term injection of constant levels of EB plus P is ineffectual in producing significantly greater growth than that produced during 19-20 days. However, it is significant that during 50 days injection period, DNA remained above level observed on 19th day of pregnancy, indicating that this hormonal treatment prevented involutionary changes. It would appear that other hormones, or other levels of these 2 steroids may be involved in production of greater growth than that observed during pregnancy. That greater growth may be produced over a 19 day period by synergism of thyroxine and growth hormone with above steroids has been shown(2,3).

It was noted that 3-me reduced mammary gland development by approximately 25% in animals receiving either 1 μ g EB plus 3 mg P or double that amount.

Levels of 3-me used were observed to produce carcinogenic changes in intact animals (5). Present study indicates that levels of hormone injected in the 3-me treated ovariectomized animal prevented observable tumor development.

Summary. Administration of 1 μ g estradiol benzoate plus 3 mg progesterone into ovariectomized rats for 20 days produced mammary gland DNA levels comparable to those observed in rats on 19th day of pregnancy, while continued injection for periods of 30, 40 and 50 days did not significantly increase observed growth. However, no involutionary changes were noted with long-term injection, indicating a maintenance of the lobule-alveolar system. Administration of 10 mg 3-methylcholanthrene twice weekly for 7 weeks reduced mammary gland growth by approximately 25% in ovariectomized rats given 1 μ g estradiol benzoate plus 3 mg progesterone or twice this amount. These hormones prevented development of observable mammary tumors.

1. Moon, R. C., Griffith, D. R., Turner, C. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v101, 788.
2. Moon, R. C., Turner, C. W., *ibid.*, 1960, v103, 149.
3. Moon, R. C., *J. Animal Sci.*, 1960, v19, 1330. (abst.).
4. Wada, H., Turner, C. W., *J. Dairy Sci.*, 1959, v42, 1198.
5. Huggins, C., *et al.*, *J. Exp. Med.*, 1959, v109, 25.

6. Dao, T. L., Sunderland, H., *J. Nat. Cancer Inst.*, 1959, v23, 567.

7. Griffith, D. R., Turner, C. W., *Proc. Soc. Exp.*

BIOL. AND MED., 1961, in press.

Received February 13, 1961. P.S.E.B.M., 1961, v106.

Reaction of Rheumatoid Sera with Fragments of Papain-Digested Rabbit Gamma Globulin.* (26488)

JOEL W. GOODMAN (Introduced by Ernest Jawetz)

Department of Microbiology, University of California Medical Center, San Francisco

Sera from a large proportion of patients suffering from rheumatoid arthritis react with rabbit gamma globulin as demonstrated by hemagglutination(1,2) and quantitative precipitin(3,4) technics. The reactant material in these sera has been termed "rheumatoid factor" and has the physicochemical properties of a gamma macroglobulin(5).

Porter(6) chromatographed a papain digest of rabbit gamma globulin on carboxymethylcellulose and obtained 3 fractions. These fragments (designated Fractions I, II, and III in the order of chromatographic elution) all had sedimentation constants of about 3.5S and molecular weights of about 50,000, 50,000, and 80,000, respectively(7). Fractions I and II were similar in chemical and biological properties. When antibody globulin was digested, each had the power to combine with, but not to precipitate, the homologous antigen. Fraction III showed no antibody activity but appeared to carry most of the antigenic determinants of the native molecule as determined by its ability to precipitate with antisera to rabbit gamma globulin.

In view of this localization of the antigenic determinants of rabbit gamma globulin on a fragment comprising about 40% of the intact molecule, it was of interest to determine which fragment or fragments of the papain digest were involved in reaction with rheumatoid factor. This would serve as a first step in comparing the structural units of the globulin molecule involved in reaction with rheumatoid factor and with specific antibody to rabbit gamma globulin.

Materials and methods. γ -Globulin. Rabbit gamma globulin (RGG) was prepared by sodium sulfate precipitation according to the method of Kekwick(8). Paper electrophoresis of the preparation demonstrated a single component with the mobility of γ -globulin.

Crystalline papain[†] was prepared from crude enzyme powder and crystallized as the inactive mercury dimer(9). It was lyophilized and stored as the dimer.

Sera. A pool of sera from rheumatoid arthritis patients was kindly provided by Dr. Wallace V. Epstein, Dept. of Medicine, Univ. of California, San Francisco. The titer of this rheumatoid pool (RP) for tanned sheep erythrocytes coated with human γ -globulin (Cohn Fraction II) was 1:7000. In 3 experiments using tanned sheep RBC coated with RGG, the titer was found to be 1:896 to 1:1792.

Mice were immunized[‡] with Fraction I of papain-digested RGG. The antigen was incorporated into a medium consisting of equal volumes of saline and Freund's adjuvant. Mice received 3 injections, each containing 1.0 mg of Fraction I. They were bled 10 days after last injection and the sera pooled.

Prior to use in experiments, all sera were incubated at 56°C for 30 minutes to inactivate complement and absorbed with an equal volume of washed packed sheep RBC for 18 hours at 4°C to remove heterophile antibody activity.

[†]Mercuripapain was prepared while the author was at the laboratory of Dr. R. R. Porter, Nat. Inst. for Medical Research, London, England.

[‡]Mice were immunized and bled by Dr. Leon S. Kind, Dept. of Microbiology, Univ. of California, San Francisco.

* Supported by grants from Nat. Science Foundation and Research Committee, Univ. of California.

Digestion and chromatography. RGG was digested with one percent mercuripapain and fractionated on carboxymethylcellulose (CM-cellulose) (10,11) as described by Porter(6). Three chromatographic peaks were obtained, corresponding to Porter's Fractions I, II, and III (FI, FII, and FIII). For one experiment, FII was rechromatographed on CM-cellulose using 0.01 M sodium phosphate buffer, pH 6.0. It was found that FII was eluted from the column with this buffer whereas FIII was retained, thus effecting a complete separation of these 2 components. FIII could be eluted with 0.2 M phosphate buffer, pH 7.0. Protein concentrations were determined by reading the absorption at 280 and 260 $m\mu$ in a spectrophotometer.

Hemagglutination tests. The capacity of RP and the mouse anti-FI pool to agglutinate sheep RBC coated with RGG and the fractions was determined. Erythrocytes were treated with tannic acid and coated with RGG or one of the fractions according to Heller *et al.*(12). Protein solutions were absorbed with washed sheep RBC prior to coating tanned cells in order to remove heterophile antibody activity. One-half ml of serial 2-fold dilutions of serum and 0.5 ml of a 0.25% suspension of coated erythrocytes were mixed in the tests. Dilutions were made with buffered saline, pH 8.0, in assays with RP. It was found that the mouse pool did not give stable endpoints in saline so in assays with this serum the 0.25% suspension of RBC was made up with buffered saline containing 2% bovine serum albumin(13). Hemagglutination endpoints were stable under these conditions. Degree of hemagglutination was read as 4+ (very strong) to— (absent) from the settling pattern of the cells following overnight incubation at 4°C. Control tubes containing tanned, uncoated RBC plus serum were included in each experiment. These never showed detectable agglutination.

Hemagglutination-inhibition tests. The capacity of RGG and of the fractions to inhibit agglutination of RGG-coated cells by RP was determined. A dilution of RP giving 4+ agglutination was used in these assays. Varying amounts of the inhibitors were

TABLE I. Hemagglutination of Coated Cells by Rheumatoid Pool.

Coated RBC	Titer
γ -Globulin	1:896 1:896
Fraction I	<1:28 <1:28
" II	<1:28 <1:28
" III	1:448 1:448

added to tubes containing 0.5 ml of RP. One-half ml of a 0.25% suspension of coated cells was then added to each tube, giving a total volume of 1.5 ml. Control tubes containing the following were included in each experiment: 1) coated RBC plus RP; 2) tanned, uncoated RBC plus RP; 3) coated RBC plus inhibitor. The latter two always failed to show detectable hemagglutination. The rest of the procedure was as described above.

Results. Table I gives the results of hemagglutination tests of RP with cells coated with RGG and the fractions. Titers are shown for 2 independent determinations. RGG-coated cells and FIII-coated cells were agglutinated by serum dilutions of 1:896 and 1:448, respectively. The titers for cells coated with FI and FII were less than 1:28, this being the lowest dilution of RP tested.

To demonstrate that the negative results obtained in hemagglutination tests of RP with FI and FII-coated cells were not due to inability of these fractions to coat tanned RBC, hemagglutination tests using coated cells and the mouse anti-FI pool were performed. The titers obtained in 2 experiments are given in Table II. Cells coated

TABLE II. Hemagglutination of Coated Cells by Mouse Anti-FI Serum.

Coated RBC	Titer
γ -Globulin	1:3584 1:3584
Fraction I	1:3584 1:3584
" II	1:3584 1:3584
" III	1:896 1:896

TABLE III. Inhibition by RGG and Fractions of HA of RGG-Coated Cells by Rheumatoid Pool.

Amt of inhibitor (mg)	Inhibitor				
	γ -Glob.	FI	FII	FII*	FIII
	Hemagglutination readings				
.001	+4, +4	+4, +4	+4, +4	+4	+4, +3
.002					+4
.004					+3
.008					+
.01	+2, +2	+4, +4	+4, +4	+4	—, —, —
.05	—, —	+4, +4	+3, +3	+3	—, —, —
.10	—, —	+4, +4	+2, +2	+2	—, —
.50	—, —	+4, +4	—, +	±	—, —

* Fraction II rechromatographed on CM-cellulose column, pH 6.0.

with FI and FII were as strongly agglutinated as cells coated with RGG, the titer being 1:3584 in each case. Cells coated with FIII were agglutinated to a titer of 1:896. It had previously been shown that a rat antiserum to FII cross-reacted very strongly with FI but only weakly with FIII(6,7), indicating that FI and FII are very similar antigenically and almost but not completely distinct from FIII.

The results of experiments to determine the effectiveness of RGG and the fractions in inhibiting agglutination of RGG-coated cells by RP are given in Table III. FIII was a more effective inhibitor than RGG on a weight basis, 0.01 mg of the former causing complete inhibition while this amount of the latter produced only partial inhibition. On a molar basis, they might prove very similar in inhibiting capacity. It is not possible, however, to make critical quantitative comparisons on the basis of hemagglutination readings. The inability of FI to cause detectable inhibition and the low inhibiting capacity of FII over the concentration range tested indicates that the former is not at all involved and the latter only weakly involved in reaction with rheumatoid factor. The possibility existed that the weak inhibiting activity of FII was due to contamination with a small amount of FIII as separation of these 2 chromatographic peaks was incomplete under the conditions employed(6). Although precaution was taken to minimize cross-contamination by discarding the effluent in the overlap area of the chromatogram, in order to make certain that contamination was not

responsible, FII from a CM-cellulose column developed at pH 6.0 was used in one experiment. Table III shows that weak inhibiting capacity was still present and appears, therefore, to be an inherent property of FII.

Discussion. The results show that most of the reactivity of RGG with RP is associated with FIII of the papain-digested molecule. Erythrocytes coated with FI and FII were not agglutinated by RP whereas cells coated with RGG and FIII were strongly agglutinated. FI was unable to inhibit agglutination of RGG-coated cells by RP. FII was able to inhibit agglutination at higher concentrations but was a much less effective inhibitor than either FIII or RGG. The results suggest that FIII and RGG are about equally effective inhibitors on a molar basis, which would be expected if almost all the reactive groups are located on the FIII segment of the RGG molecule. Since the inhibition tests indicate that FII does react weakly with rheumatoid factor, the absence of agglutination of FII coated RBC by RP might possibly be due to physical blockage of the structural unit involved by virtue of attachment of the molecule to the surface of the red cell. Should this be the case, it would mean that different structural units are involved in reaction with RP and with mouse anti-FI serum since the latter was capable of agglutinating FII coated cells. An alternate explanation might be that the cells were insufficiently coated to give detectable agglutination, since the reaction with RP is weak at best.

FIII has been found to be the carrier of several properties characteristic of RGG. In addition to possessing most of the reactivity of the molecule with specific antisera(6,7) and, as shown here, with rheumatoid factor, of the 3 fragments it most closely resembles the parent molecule with respect to transmission across the foetal yolk-sac membrane of the rabbit(14). The order of transmission across the membrane was found to be FIII > FII > FI, identical to the order of reactivity with RP. The configurations on the γ -globulin molecule comprising the antigenic determinants, the units involved in reaction with rheumatoid factor and those recognized by

rabbit cells as homologous γ -globulin are therefore largely located on a segment comprising about 40 percent of the parent molecule. It will be necessary to obtain much smaller active fragments of FIII in order to establish the relationships of the chemical groupings responsible for these properties.

Summary. Most of the reactivity of rabbit γ -globulin with a pool of sera from rheumatoid arthritics was found to be associated with Fraction III of the papain-digested globulin molecule. Fraction II showed a low degree of reactivity while Fraction I appeared to be completely inactive.

1. Waaler, E., *Acta Path. Microbiol. Scand.*, 1940, v17, 172.
2. Rose, H. M., Ragan, C., Pearce, E., Lipman, M. O., *Proc. Soc. Exp. Biol. and Med.*, 1948, v68, 1.
3. Vaughan, J. H., *J. Immunol.*, 1956, v77, 181.

4. Vaughan, J. H., Ellis, P. J., Marshall, H., *ibid.*, 1958, v81, 261.
5. Franklin, E. C., Holman, H. R., Müller-Eberhard, H. J., Kunkel, H. G., *J. Exp. Med.*, 1957, v105, 425.
6. Porter, R. R., *Nature*, 1959, v182, 670.
7. ———, *Biochem. J.*, 1959, v73, 119.
8. Kekwick, R. A., *ibid.*, 1950, v34, 1248.
9. Kimmel, J. R., Smith, E. L., *J. Biol. Chem.*, 1954, v207, 515.
10. Peterson, E. A., Sober, H. A., *J. Am. Chem. Soc.*, 1956, v78, 751.
11. Sober, H. A., Gutter, F. J., Wyckoff, M. M., Peterson, E. A., *ibid.*, 1956, v78, 756.
12. Heller, G., Jacobson, A. S., Kolodny, M. H., Kammerer, W. H., *J. Immunol.*, 1954, v72, 66.
13. Boyden, S. V., *J. Exp. Med.*, 1951, v93, 107.
14. Brambell, F. W. R., Hemmings, W. A., Oakley, C. L., Porter, R. R., *Proc. Royal Soc.*, 1960, v151, 478.

Received February 13, 1961. P.S.E.B.M., 1961, v106.

Rat Virus (RV) Infections in Hamsters. (26489)

LAWRENCE KILHAM

Laboratory of Virology & Rickettsiology, Division of Biologic Standards, National Institutes of Health, Bethesda, Md.

Rat virus (RV) was first recognized in rat embryo tissue cultures inoculated with materials from tumor-bearing rats. In such cultures the production of specific cytopathogenic effects (CPE) and of hemagglutinins indicated activity of the virus(1). Preliminary tests in animals failed to reveal pathogenic properties of RV. The present report describes (a) a fatal disease induced by RV in hamsters of one to 4 days of age, (b) occurrence of virus and viral hemagglutinins in the tissues and fluids of these animals and (c) spread of virus to uninoculated hamsters. Sucklings which happen to survive the acute disease may develop a stunted growth resembling mongolism. This dwarfism has been reported previously(2).

Materials and methods. Modifications and extensions of procedures not previously described(1) are given below.

Virus. The strain of virus used throughout current work was RV-13, which was car-

ried through approximately 12 rat embryo tissue cultures and 4 passages in suckling hamsters prior to a final passage in rat embryo tissue culture. This tissue culture seed virus lot which was stored at -40°C , had an HA titer of 1:512 and an LD_{50} of 10^{-5} as measured by deaths induced in 1-day old suckling hamsters within a period of 10 days. As described below, a 10^{-6} dilution of the seed induced dwarfism but no acute fatal disease.

Inoculation and harvesting of hamsters. Syrian hamsters from the Animal Production Center of the NIH were inoculated as sucklings by the intracerebral route. Uninoculated control animals were retained in each litter tested; controls were identified by clipping a portion of their tails. Animals harvested at various stages of infection were anaesthetized, decapitated and exsanguinated into a dish moistened with heparin. Bladders were then clamped and incised in order

TABLE I. Effects of Rat Virus (RV) in Suckling Hamsters of Different Ages.

		Age of hamsters at time of inoculation*			
		<24 hr	4 days	5 days	6 days
Conditions induced by RV	Acute fatal disease	56/56	35/47	None	None
	Mongolism	None	12/47	"	"
	Latent infection	"	None	2/2	2/2
	Tissues†	Kidney	Kidney	Liv. & kid.	Liv. & kid.
Titrations	HA	81,920	40,960	1280	0
	Infectivity	7.3‡	7.3	7.0	4.8

* Hamsters inoculated intracer. with 10^4 LD₅₀ of RV from 4th hamster passage.

† Tissue pools were from 2 to 3 hamsters, killed 5 days after inoculation.

‡ Titers expressed as inverse log of dilution killing 50% of hamsters <24 hr old.

to collect urine. A usual procedure was to freeze all specimens immediately after harvesting and to store them at -40°C until testing was undertaken. All specimens for determination of presence of virus were finally made into 1:10 suspensions with medium 199 containing 10% calf serum, plus penicillin and streptomycin, and clarified by repeated centrifugations at 2500 rpm.

Infectivity titrations. Titrations of tissue suspensions and other materials for infective virus were performed by intracerebral inoculation of hamsters less than 24 hours of age with serial, 10-fold dilutions. The animals were then observed over a period of 10 days and 50% end-points, based on deaths and survivals, were calculated by the method of Reed and Muench(3).

Hemagglutination (HA). In these tests, the specimens of tissue and body fluids, clarified by centrifugation as described above, were carried through serial 2-fold dilutions in buffered saline (pH 7.4) in volumes of 0.4 ml. An equal volume of a 0.5% suspension of washed guinea pig erythrocytes was added and the cells were allowed to settle for one hour at room temperature. Titers were then read from patterns formed by the erythrocytes at the bottoms of the tubes. Results of these HA titrations were expressed in terms of actual dilutions of the virus suspensions prior to addition of red cells.

Serologic tests were of 2 types. In hemagglutination-inhibition (HI) tests, serial 2-fold dilutions of serum were made in buffered saline, in volumes of 0.2 ml and 8 to 16 HA units of RV were added in equal volumes. After this mixture had incubated for

$\frac{1}{2}$ hour at 36°C , 0.4 ml of 0.5% of guinea pig erythrocytes were pipetted into each tube. Results were read by the pattern method after one hour at room temperature. As in the HA tests, final results were read in terms of actual dilutions of the material tested prior to addition of other constituents, i.e. of virus and RBC. Neutralization tests (NT) were performed with equal volume mixtures of undiluted serum and of virus suspension containing 10 to 100 LD₅₀'s of RV; such mixtures, after $\frac{1}{2}$ hour of incubation at 36°C , were inoculated intracerebrally into hamsters less than 24 hours of age. All sera used, in both types of serologic tests, HI and NT, were inactivated by heating at 56°C for $\frac{1}{2}$ hour.

Results. Infection and disease. RV can produce acute fatal disease, a stunted form of growth resembling mongolism, or a latent infection, depending on dosage and age of the hamsters at time of intracerebral inoculation. These effects are outlined in Table I. The acute disease, induced in sucklings of from less than one to 4 days of age by 10^4 LD₅₀ of RV was manifested on the 4th to the 8th day by a slight anal exudate, a dark red color of the intestinal tract visible through the skin of the abdomen, and generalized weakness. Sucklings with these signs usually died within hours, with stomachs full of milk. Some of the hamsters in the group inoculated at 4 days of age had a severe anemia when moribund 7 to 8 days after inoculation. Thus only a small amount of thin, watery blood could be obtained when these animals were decapitated under anaesthesia.

A stunted form of growth associated with

TABLE II. Results of HA and Infectivity Tests for Presence of Virus in Tissues of Suckling Hamsters Inoculated with RV When 4 Days of Age.

Day after inoc.*	Titers of tissue suspensions†											Comment	
	HA						Infectivity for hamsters IC						
	Liv.	Kid.	Gut	Brain	Blood	Urine	Liv.	Kid.	Gut	Brain	Blood		Urine
2	None‡	None			None	None	<1 §				4.2	None	Well
4	20480	20480		80	"	"	6	6		3.6	5.7	3.6	"
5	40960	40960		640	"	"	7	7.3			5.8	3.8	Some mor- tality
6	20480	10240		640	"	80	7.6	7.6		4.8	5.7	5.6	Max mor- tality
7	2560	1280	2560				5.5	5.1	5.6				
8	None	None	None				3.6	5	5.5				Moribund

* Hamsters inoculated IC with 10^4 LD₅₀ of RV.

† Tissues from 2 to 3 hamsters pooled for each determination.

‡ None: For HA test, means no hemagglutination at a starting dilution of 1:80. For infectivity test, means no virus isolated from a 1:10 suspension.

§ Titers expressed as inverse log of dilution killing 50% of hamsters <24 hr old.

defective incisor teeth was a second type of disease encountered among the sucklings inoculated as 4 day-olds. This form of disease has been described in newborn hamsters inoculated with sublethal amounts of RV(2).

Latent infection, a third condition induced by RV, was encountered in hamsters receiving RV at 5 or 6 days of age. Hemagglutinins were demonstrable in tissues from sucklings inoculated when 5 days old, but not in those inoculated at 6 days of age (Table I). Infective virus was recovered from hamsters of both age groups but the amount detectable in the older animals was less.

RV has been carried through 20 continuous passages in suckling hamsters. Tests similar to those done with 4th passage hamster virus (Table I) were undertaken with 10th passage hamster virus; the results were comparable. Most of these passages were made by means of 10% suspensions of pooled livers and kidneys, given by the intracerebral route. Intraperitoneal, subcutaneous and intranasal inoculations have also proved to be effective in transmission. In the later part of the work the inocula were held at 65°C for 15 minutes on an assumption that this heating might eliminate adventitious agents, if such were present. Such heating has had no apparent effect on the infectivity of RV.

Hemagglutinins and infective virus. The pathogenesis of RV-infections in suckling hamsters can be followed to some extent by

testing various tissues and body fluids for amounts of RV present on successive days after inoculation. Table II gives results of 2 types of viral titrations on materials from sucklings inoculated as 4-day-olds and sacrificed 2 to 8 days later.

It is apparent from the tabular data that viremia was detected as early as the 2nd day by means of infectivity titration but circulating hemagglutinins were not demonstrated even on the 5th day when intensity of the viremia was at its peak and when hemagglutinins reached their maximum titer in the liver and kidney. Virus as determined by both procedures was present in largest amounts in liver and kidney but occurred also in brain, gut and urine. There was only a rough correlation between the results obtained by the 2 titration procedures. When hemagglutinins were demonstrable in a specimen the infective titer of that material was usually in excess of 10^{-5} . However, not all specimens with such amounts of infective virus gave positive results in the hemagglutinin tests, *viz.*, blood. Neither hemagglutinins nor infective virus were demonstrated in tissues, urine or blood of 2 groups of normal unexposed hamsters sacrificed when 5 and 10 days of age respectively.

Spontaneous transmission of RV. Data presented in Table II suggested that RV was shed into the environment from both urinary and intestinal tracts and raised the question

TABLE III. Spontaneous Transmission of RV among Hamsters.

Exposure to RV infected litter	Age group	Uninoculated hamsters			HI-antibodies		
		No. animals in group	Duration of exposure	Disease	No. sera tested	No. positive	Range of titers
Direct (in same jar)	Newborn	9	6-9 days	Fatal 6-9 days	None	—	—
	4 days old	12	3 wk	None	6	6	320-1280
	Mothers (whose y'ng were inoc.)	8	3 "	"	8	8	160-1280
Long, indirect (same room); transient, direct (ate inoc. young)	Mother†	5	1-2 days	"	5	None*	
	" †	4	3 wk	"	4	"	

* None: No hemagglutination at starting dilution of 1:80.

† These mothers lost their young, through cannibalism, within a few days of birth.

as to the extent to which spontaneous transmission might take place. Table III shows that such transmission occurs. In one experiment involving litters of newborn hamsters, 9 of the 9 uninoculated controls developed the same type of fatal illness as their inoculated littermates. However, the former died within 6 to 10 days while the latter succumbed in 4 to 5 days. Pooled livers and kidney tissues from several of the spontaneously infected hamsters had HA titers of 1:10,240. In contrast, uninoculated animals among the 6 litters which were injected when 4 days of age, remained well even though they became infected as evidenced by the development of HI antibodies (Table III). Table III also shows that RV spread, spontaneously, to the mothers of infected litters. Although these adults displayed no signs of infection, they had circulating HI antibodies when tested 3 weeks after inoculation of their young. Postpartum hamsters, nevertheless, do not readily acquire infection from brief, direct exposure to animals having RV infections. Thus 4 mothers which ate their babies within a day after inoculations with RV and subsequently lived in the same room with infected families for 3 weeks, failed to develop RV antibodies (Table III). The 5 mothers mentioned in Table III which had no RV antibodies at the time their young died several days after inoculation merely add to the experience, throughout these studies, that adult hamsters do not ordinarily possess RV antibodies.

Some attributes of hemagglutinins. The hemagglutinins recovered from infected hamster tissues appeared to be specific for rat virus. Thus they had the same general characteristics of hemagglutinins produced by RV in rat embryo tissue culture *i.e.*, they agglutinated guinea pig erythrocytes at room temperature without elution. Secondly, guinea pig antisera prepared against RV inhibited the hemagglutinins from infected hamster tissue in high titer. An illustrative experiment is summarized in Table IV. Sera from guinea pigs prior to immunization inhibited HA in low titer. This apparently non-specific inhibition of RV hemagglutination at low titers has been demonstrated in normal sera from a number of species. Its exact nature is not clear.

Discussion. RV is a virus which has caused no apparent disease on inoculation into rats, its natural host, or into mice which are the host of polyoma virus, a seemingly related agent(1,2). Hamsters are susceptible to both viruses, however, and the diseases induced offer points of contrast. One difference is the failure of RV to induce neoplasms. It is conceivable that RV is too lethal in its effects on hamster tissue to achieve that "delicate balance—between virulence of virus and resistance of host," as phrased by Stewart(4) which is possibly essential if a virus is to induce cancer. Another contrast between RV and polyoma is found in their patterns of multiplication in suckling hamsters. Rowe and associates(5)

TABLE IV. Specificity of Hemagglutinins Found in Tissue of RV-infected Hamster.

Guinea pig serum	HA units of RV-infected liver susp.	Dilutions of guinea pig serum								
		20	40	80	160	320	640	1280	2560	5120
Pre-inoc.	16	0	+	+	+	+	+	ND	ND	ND
	8	0	0	+	+	+	+	"	"	"
	4	0	0	0	+	+	+	"	"	"
3 wk post-inoc.*	16	0	0	0	0	0	0	+	+	+
	8	0	0	0	0	0	0	0	+	+
	4	0	0	0	0	0	0	0	0	+

* Guinea pig immunized with RV-infected rat embryo tissue culture fluid.

have shown that polyoma virus reaches peak titers in 4 to 6 days after inoculation, but titers of infective virus are low, hemagglutinins fail to appear and there is no immediate disease or subsequent dwarfism. Neoplasms are the characteristic signs of polyoma infection in infected hamsters. The multiplication of polyoma virus in suckling mice (4) follows patterns which are closer to those described above for rat virus in suckling hamsters. Thus Rowe *et al.*, encountered infective virus in titers of 10^{-6} and 10^{-7} in kidneys and livers of infected animals and these were associated with presence of hemagglutinins. Polyoma virus was also demonstrable in both blood and urine. The fact that this agent can induce dwarfism associated with defective incisor teeth in mice is an additional point of similarity with RV (2).

Summary. 1) Rat virus (RV) can induce 3 different conditions in suckling hamsters according to dose of virus and age of the animal at time of inoculation. These conditions are (a) a fatal disease in sucklings 1 to 4 days of age; (b) a stunted growth in those

surviving a minimal dose, and (c) a latent infection of older sucklings. 2) Spontaneous transmission of RV can take place from inoculated to uninoculated hamsters. 3) Infected sucklings have infective virus in high titer in livers, kidneys and other organs as well as in lesser amounts in blood and urine. Specimens with the highest infectivity titers have also had considerable amounts of hemagglutination activity. 4) Rat virus has been carried through 20 passages in suckling hamsters. Intracerebral, intranasal, subcutaneous and intraperitoneal routes of inoculation have all been effective in inducing acute disease.

1. Kilham, L., Olivier, L. J., *Virology*, 1959, v7, 428.
2. Kilham, L., *Virology*, 1961, v13, 141.
3. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
4. Stewart, S. E., *Sci. Am.*, 1960, v203, 63.
5. Rowe, W. P., Hartley, J. W., Estes, J. P., Huebner, R. J., *Nat. Cancer Inst. Monograph*, 1960, No. 4, 189.

Received February 15, 1961. P.S.E.B.M., 1961, v106.

Studies of Placental Morphogenesis I. Radioautographic Studies of Human Placenta Utilizing Tritiated Thymidine.* (26490)

RALPH RICHART (Introduced by George Margolis)

Department of Pathology, Medical College of Virginia, Richmond

The manner in which the syncytiotrophoblast takes origin and is maintained has occupied the attention of many investigators interested in the placenta. This paper pre-

sents preliminary data from investigations of this problem.

Method. Fresh sterile human placental tissue was obtained from 4 therapeutic abortions ranging in gestational age from 14 to

* Supported by USPHS Grant No. SG 13975.

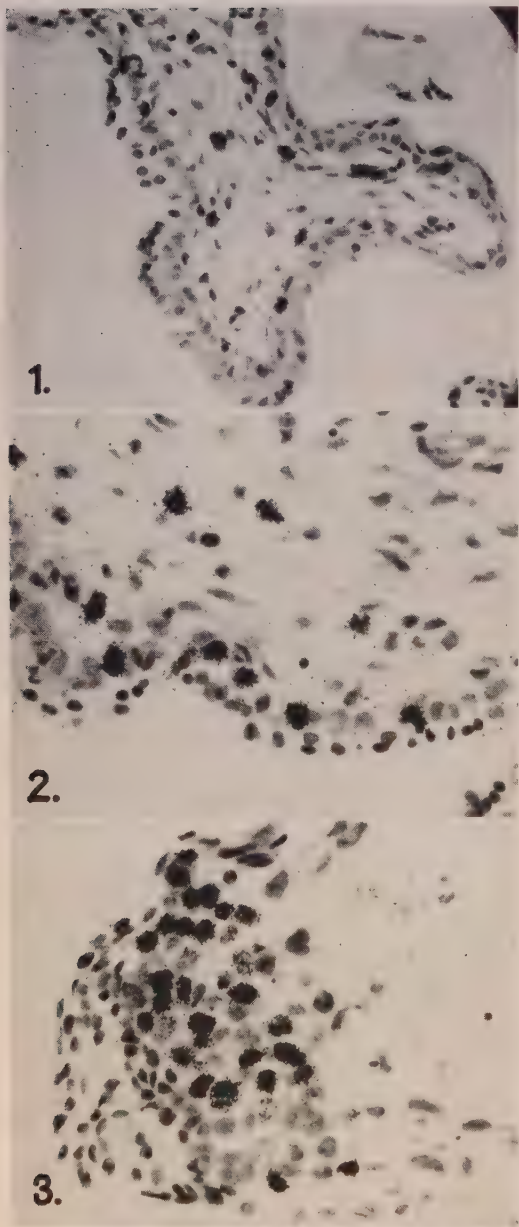


FIG. 1. Radioautograph of human placenta. Note labeled nuclei among cells of cytotrophoblast, villous mesenchyme, and vascular endothelium.

FIG. 2. Radioautograph of human placenta with many labeled nuclei among cells of cytotrophoblastic layer but no labeled nuclei among syncytiotrophoblastic cells.

FIG. 3. Radioautograph of human placenta with intense activity among nuclei of cytotrophoblastic column but no labeled nuclei in peripheral syncytiotrophoblast.

18 weeks. Full thicknesses of placental tissue were placed in Puck's F4FC tissue culture media to which had been added tritiated thymidine of $1.9 \mu\text{C}$ per millimole specific activity at concentrations ranging from 1 to $12 \mu\text{C}$ per milliliter of media. The cultures were maintained at $37 \pm 0.2^\circ\text{C}$ with air as the gas phase and pH was adjusted daily to 7.2. Samples of the placental tissue were removed from incubation, washed in balanced salt solution and fixed in neutral buffered formalin at intervals ranging from 1 to 72 hours. The tissues were prepared for sectioning, cut at 6 μ , and mounted on glass slides. The sections were deparaffinized in xylol, hydrated and dipped in undiluted Kodak liquid emulsion type NTB 3, and exposed at 4°C for 3 to 4 weeks in bakelite boxes containing Drierite. They were then developed in Kodak *D-19* developer, stained with Harris' hematoxylin, coverslipped, and examined microscopically.

Results. In more than 200 slides examined, each containing from 8 to 12 sections, unequivocal evidence of thymidine uptake by syncytiotrophoblastic nuclei was not encountered. There was abundant activity in nuclei of the villous mesenchyme and vascular endothelium as well as the Langhans cytotrophoblast layer (Fig. 1, 2). There was particularly heavy activity in the nuclei of the cytotrophoblastic columns (Fig. 3).

Discussion. The problem of how the syncytiotrophoblast takes origin has been discussed by Hertig *et al.*(3) Wislocki and Bennett(5) and Wimsatt(4), all of whom proposed an amitotic form of division as one hypothesis to account for the quantitative increase in the syncytium without morphologically distinguishable mitotic activity. Hamilton and Boyd(2) also offered amitotic division as a possible mechanism for the origin of the syncytiotrophoblast.

Our failure to demonstrate thymidine uptake by syncytial nuclei argues strongly against amitotic division in the syncytiotrophoblast, at least in the human placenta under the conditions of our experiments. It may be argued that amitotic divisions could occur in absence of new DNA synthesis and

hence without thymidine uptake but this is unlikely in the case of human syncytiotrophoblast in view of the near-diploid DNA values found by Galton(1) in these nuclei using microspectrophotometry.

On the basis of these experiments it now seems likely that the syncytiotrophoblast is a mitotic end stage that is continually derived from another cell type, possibly cytotrophoblast. Experiments are under way to investigate this possibility and to extend our findings to other species and other experimental conditions.

Summary. Portions of human placental tissue have been studied by radioautography utilizing tritiated thymidine. Thymidine uptake could be demonstrated in cytotropho-

blast, villous mesenchyme and vascular endothelium but not in the syncytiotrophoblast. This finding suggests that amitotic division of the syncytiotrophoblast does not play a prominent role in the derivation of this cellular layer in the human placenta of 14-18 weeks gestational age.

1. Galton, M., personal communication.
2. Hamilton, W. J., Boyd, J. D., *J. Anat.*, 1960, v94, 297.
3. Hertig, A. T., Rock, J., Adams, F. C., *Am. J. Anat.*, 1956, v98, 435.
4. Wimsatt, W. A., *ibid.*, 1945, v77, 1.
5. Wislocki, G. B., Bennett, H. S., *ibid.*, 1943, v73, 335.

Received February 17, 1961. P.S.E.B.M., 1961, v106.

Effects of Time and Steroid Concentration on Binding of C^{14} -Estrogens in Human Plasma.* (26491)

AVERY A. SANDBERG, W. R. SLAUNWHITE, JR. AND HARRY N. ANTONIADES
(Introduced by E. A. Mirand)

Roswell Park Memorial Institute, Buffalo, N. Y., and Protein Foundation Laboratories, Jamaica Plain, Mass.

We have reported(1-3) on the distribution of radioactive steroids and their metabolites among the various plasma protein fractions following intravenous injection of C^{14} -steroids into human subjects. Thus, it was shown that most unconjugated and conjugated steroids and their metabolites were bound principally to the plasma fractions IV-1 and V. In the case of C^{14} -estrone and C^{14} -estradiol the bloods were drawn 20 and 45 minutes after injection, respectively. This study deals with the effects of time of blood withdrawal and prior administration of large amounts of non-radioactive steroids, either identical to or different from the radioactive steroids, on distribution of radioactivity among the fractionated plasma proteins.

Experimental. The materials, methods and experimental procedures were similar to those previously reported(1,2) with the following exceptions: 1) *ca.* 250 ml of blood

was withdrawn 30 minutes after injection and an equal volume 90-150 minutes later and 2) two subjects, in addition to receiving radioactive steroid, were infused a second time with the same radioactive steroid plus 50 mg of non-radioactive steroid. The solution for infusion was prepared by dissolving 50 mg of crystalline U.S.P. steroid in 10 ml of ethanol, which was added dropwise with stirring to 240 ml of 1% HSA (human serum albumin) resulting in the appearance of a very fine microcrystalline suspension of the steroid. This solution was infused intravenously over 30 minutes. The C^{14} -steroid (2.7 μ C/mg) dissolved in 50 ml of 4% ethanol in saline, was injected intravenously over a period of 2-3 minutes through the tubing half-way through the infusion. The timing commenced with injection of the radioactive steroid.

Before discussing the results, it may be worthwhile to comment on the state of the carrier and radioactive steroids injected in

*Supported in part by grants from U.S.P.H.S.

TABLE I. Distribution of Bound and Unbound Radioactivity in Human Plasma after Injection of Ca 1 μ c of C¹⁴-labeled Steroids with and without Unlabeled Steroid.

Steroid inj.	Phlebotomy time (min.)	Radioactivity	Bound (fractions I-V)			Unbound (supernatant fluid V)		
			Total	Conjugated	Unconjugated	Total	Conjugated	Unconjugated
16-C ¹⁴ -estrone (M.F.)	30	c/m/100 ml	7140	4370	2770	7470	4400	3070
		%	49	61*	39*	51	59*	41*
	180	c/m/100 ml	5530	5260	270	4980	4530	450
		%	53	95	5	47	91	9
16-C ¹⁴ -estrone + 50 mg progesterone (M.F.)	30	c/m/100 ml	5870	3920	2050	5650	3000	2650
		%	51	65	35	49	53	47
	180	c/m/100 ml	4750	4440	310	5160	4680	480
		%	48	93	7	52	91	9
16-C ¹⁴ -estradiol-17 β (R.C.)	30	c/m/100 ml	4820	4130	690	7330	6340	1090
		%	40	86	14	60	85	15
	120	c/m/100 ml	3390	3280	110	3780	3580	200
		%	48	97	3	52	95	5
16-C ¹⁴ -estradiol-17 β (J.J.)	30	c/m/100 ml	5150	3900	1250	4720	3170	1550
		%	52	76	24	48	67	33
	120	c/m/100 ml	2510	2250	260	2830	2490	340
		%	47	90	10	53	88	12
16-C ¹⁴ -estradiol-17 β + 50 mg estradiol-17 β (J.J.)	30	c/m/100 ml	3630	2460	1170	2650	1670	980
		%	58	69	32	42	65	35
	120	c/m/100 ml	710	600	110	700	510	190
		%	50	85	15	50	73	27

* Percentages are calculated by using the value in "Total" column as 100%.

the 1% HSA (in 4% ethanol in saline). From previously published results(1) and from unpublished data in our laboratory it can be calculated that a maximum of 10 mg of estradiol-17 β and 5 mg of progesterone, out of the 50 mg administered, were bound by the administered HSA. Hence, when the C¹⁴-labeled steroid was injected half-way through the infusion, the preponderant part of the radioactive steroid was not associated with the HSA, but was in the aqueous phase. Since the amount of HSA administered over a period of 30 minutes represents about 1/50 of total plasma albumin, it can be safely presumed that the binding of most of the carrier and radioactive steroids occurred to endogenous plasma protein.

Results and discussion. The distribution of protein bound radioactive C¹⁴-estrone or C¹⁴-estradiol and their metabolites is shown in Table I and is very similar to our previous results(1). Percentage of radioactivity bound and unbound was not materially affected by time of blood withdrawal, even as late as 3 hours after injection of the radioactive steroid. In all cases the percentage of radio-

active conjugated metabolites increased with time. Since previous *in vitro* work(1,3) indicated that the binding site (or sites), at least on albumin, for progesterone was different from that for C¹⁴-estrone, 50 mg of the former steroid were given by infusion before injection of C¹⁴-estrone to ascertain whether progesterone would affect the metabolism and distribution of the C¹⁴-estrone *in vivo*. Prior injection of the large amount of unlabeled progesterone produced no pronounced changes in the binding of radioactive C¹⁴-estrone and its metabolites when compared to that following injection of C¹⁴-estrone alone.

Injection of a large amount of estradiol resulted in more rapid disappearance of conjugated, radioactive metabolites of C¹⁴-estradiol in both the bound and unbound fractions. Since very little difference was seen in concentration of either bound or unbound unconjugated radioactivity following administration of 50 mg of estradiol and C¹⁴-estradiol, the more rapid clearance of the conjugates of these steroids may be related to the saturation of the protein system responsible for the binding of such conjugates. This

TABLE II. Distribution of Radioactivity Bound to Human Plasma Fractions after Injection of Ca 1 μ c of C¹⁴-labeled Steroids with and without Unlabeled Steroid.

Steroid inj.	Phlebotomy time (min.)	Radioactivity	Conjugated					Unconjugated				
			Plasma fraction No.					Plasma fraction No.				
			I	II+ III	IV-1	IV-4	V	I	II+ III	IV-1	IV-4	V
16-C ¹⁴ -estrone (M.F.)	30	c/m/100 ml	34	370	140		3830	91	510	450		1720
		%	1	8	3		88	4	18	16		62
	180	c/m/100 ml	72	380	370	460	3980	5	55	26	15	169
		%	1	7	7	9	76	2	20	10	5	63
16-C ¹⁴ -estrone + 50 mg progesterone (M.F.)	30	c/m/100 ml	110	710	320	150	2530	38	310	360	66	1280
		%	3	19	8	4	66	2	15	18	3	62
	180	c/m/100 ml	130	210	720	87	3290	22	39	44	3	202
		%	3	5	16	2	74	7	13	14	1	65
16-C ¹⁴ -estradiol-17 β (R.C.)	30	c/m/100 ml	130	300	200	160	3340	65	140	76	19	390
		%	3	7	5	4	81	9	20	11	3	57
	120	c/m/100 ml	76	240	160	130	2670	5	23	13	8	56
		%	2	7	5	4	82	5	21	12	7	51
16-C ¹⁴ -estradiol-17 β (J.J.)	30	c/m/100 ml	43	180	690	580	2400	91	180	180	56	740
		%	1	5	18	15	61	8	14	14	5	59
	120	c/m/100 ml	64	120	89	140	1840	11	40	17	12	175
		%	3	5	4	6	82	4	16	7	5	68
16-C ¹⁴ -estradiol-17 β (J.J.)	30	c/m/100 ml	47	210	480	74	1650	37	180	210	21	821
		%	2	9	19	3	67	3	15	18	2	62
	120	c/m/100 ml	16	75	83	15	410	7	8	25	3	68
		%	3	12	14	3	68	6	7	22	3	62

Percentages represent breakdown of the total radioactivity of Fractions I to V.

would result in more rapid clearance of the conjugates from the plasma and was evidenced by the more rapid excretion of radioactivity in the urine when large amounts of carrier were given in addition to C¹⁴-estradiol (4). It would also seem to indicate that the binding system for the unconjugated metabolites was not saturated by the large amount of estradiol given. On the basis of previously published data(1) in the case of estradiol it would require 5-6 times (250-300 mg or more) of the amount of estradiol injected in the experiments reported to saturate the binding capacity of the serum albumin. Since the injected steroid is metabolized rather rapidly, it would require a relatively enormous amount of estradiol to saturate the protein binding system for this particular steroid.

Distribution of the bound radioactivity among plasma fractions is shown in Table II. The preponderant part (over 60% of the unconjugated steroid, over 70% of the conjugated steroids) of the radioactivity was in Fraction V and did not vary much with the phlebotomy time. Prior administration of unlabeled steroid produced no pronounced

changes in distribution of the radioactive steroids among the various plasma fractions. Mild increases in the amount of radioactivity associated with Fraction IV-1 at the longer phlebotomy time can be seen in 3 instances, when compared to similar circumstances without administration of unlabeled compound.

In regard to the concentration of "unbound" radioactivity in plasma (Table I), one should keep in mind that under the conditions of the fractionation techniques employed in these studies loosely protein-bound C¹⁴-estrogens might be dissociated from the protein. At least 3 factors could influence the binding: ionic strength, pH, and ethanol concentration. The conditions for separation of Fraction I, II, III and IV-1 are moderate. The conditions for separation of Fractions IV-4 (40% ethanol, pH 5.8) and V (40% ethanol, pH 4.7) might cause real concern. As we have stated(1,2), "the major error in interpretation which results from this effect of the fractionating technique has been the suggestion that an appreciable portion of infused steroid may circulate in plasma un-

bound to protein." The importance of Fraction V (albumin) in the binding and transport of estrogens in human plasma has been demonstrated(1,3,5,6). The results of these studies point to albumin as the major protein concerned with transport of estrogens and their metabolites in human plasma.

Summary. 16-C¹⁴-estrone and 16-C¹⁴-estradiol-17 β were injected intravenously into normal human subjects. C¹⁴-estrogens were injected either alone or following infusion of 50 mg unlabeled progesterone or estradiol-17 β . Blood samples were drawn at 30 minutes and at 120 or 180 minutes after injection. The blood plasma was fractionated by the cold ethanol fractionation techniques and plasma fractions were examined for radioactivity. The delay of time of phlebotomy resulted in an increase in percentage of conjugated metabolites. Injection of a large amount of estradiol-17 β resulted in a more rapid disappearance of conjugated radioac-

tive metabolites of C¹⁴-estradiol-17 β in both protein-bound and unbound forms. No pronounced changes in distribution of radioactivity among the various plasma fractions occurred as a result of prior administration of large amounts of unlabeled steroid.

We wish to acknowledge the capable assistance of Mr. Lawrence Beecher and Mrs. Maria Karsay.

1. Sandberg, A. A., Slaunwhite, W. R., Jr., Antoniadis, H. N., *Rec. Prog. Hormone Res.*, 1957, v13, 209.
2. Antoniadis, H. N., Pennell, R. B., Slaunwhite, W. R., Jr., Sandberg, A. A., *J. Biol. Chem.*, 1957, v229, 1071.
3. Slaunwhite, W. R., Jr., Sandberg, A. A., *Endocrinology*, 1958, v62, 283.
4. ———, in preparation.
5. Bischoff, F., Stauffer, R. D., *Am. J. Physiol.*, 1957, v191, 313.
6. Slaunwhite, W. R., Jr., Sandberg, A. A., *J. Clin. Invest.*, 1959, v38, 384.

Received February 20, 1961. P.S.E.B.M., 1961, v106.

Evidence for a Central Mechanism in Angiotensin Induced Hypertension.* (26492)

ROBERT K. BICKERTON AND JOSEPH P. BUCKLEY (Introduced by L. V. Beck)
*Cardiovascular Research Laboratories, Department of Pharmacology, School of Pharmacy,
University of Pittsburgh, Pittsburgh, Pa.*

Ever since Goldblatt(1) successfully produced sustained hypertension in dogs by partially constricting both renal arteries, investigators have been trying to isolate pressor substances from the blood of hypertensive animals and patients. Angiotensin II(2), a pressor octapeptide has been isolated in renal hypertensives(3) and has been synthesized by Bumpus *et al.*(4,5). Most investigators (4-6) have felt that the hypertensive properties of Angiotensin II were due entirely to the peripheral actions of the compound and have inferred that this agent did not act through the central nervous system. The purpose of this study was to determine if Angio-

tensin II possessed central hypertensive properties, and if so determine the possible mechanism of action.

Method. Six dog cross circulation preparations, modified after that described by Taylor and Page(7), were utilized in this study. Mongrel dogs were anesthetized by an intravenous injection of 35 mg/kg of pentobarbital sodium. The external jugular veins and common carotid arteries were isolated and covered with gauze moistened in normal saline solution. The internal jugular veins were doubly ligated and cut between the ligatures. The neck musculature was separated into sheets and removed, utilizing electrocautery, to expose the vertebral column from C-2 to C-5. A dorsal laminectomy, which included removal of the spinous processes of the more

*Supported in part by grants from Nat. Inst. Health, and from Ciba Pharmaceutical Products, Inc., Summit, N. J.,

TABLE I. Effects of Angiotensin II on Donor and Recipient Blood Pressures in the Dog Cross Circulation Preparations.

Exp. No.	Dose (μ g/kg)	Route	Donor					Recipient				
			Wt (kg)	Sex	Original B.P.† (mm Hg)	Rise in B.P. (%)	Duration (min.)	Wt (kg)	Sex	Original B.P.† (mm Hg)	Rise in B.P. (%)	Duration (min.)
1	1.0	IA	11.3	♂	125	40.0	4.0	8.6	♂	135	11.1	.5
	1.0	"			120	37.5	3.0			130	19.2	.5
	1.0	"			130	23.0	3.0			115	13.0	.5
	1.0	IV-R			130	.0	.0			115	52.1	6.0
2	.4	IA	11.0	♂	130	38.4	4.0	11.2	♀	140	14.2	1.0
	.4	"			125	24.0	4.0			140	14.2	.5
	.4	"			130	15.3	1.0			140	10.7	1.0
	.8	"			125	20.0	1.0			140	10.7	1.0
	1.2	"			150	33.3	2.0			125	20.0	1.0
	1.2	IV-R			150	.0	.0			125	60.0	2.5
	1.2	IA*			150	20.0	1.5			90	44.4	1.0
3	1.2	IA	16.6	♂	150	66.6	5.0	12.7	♀	200	25.6	5.0
	1.2	" *			100	50.0	3.0			200	12.5	2.0
4	2.0	"	10.5	♀	125	12.0	2.0	6.7	♀	80	25.0	.5
	2.0	"			150	§	§			135	14.0	1.0
	2.0	"			150	§	§			130	19.2	1.0
	2.0	"			140	§	§			70	42.8	1.0
5	1.0	"	23.7	♂	100	100.0	5.0	12.0	♂	100	50.0	.5
	1.0	"			100	100.0	5.0			100	50.0	.5
	1.0†	"			100	100.0	5.0			50	0.0	.0
6	1.0	"	§	§	90	78.0	5.0	§	§	90	50.0	1.0
	1.0	"			100	90.0	4.0			110	45.4	.7
	1.0†	"			75	86.6	4.0			65	7.7	.5
	.5	IV-R			60	.0	0.0			60	109.0	6.0

* Vagi were sectioned in cervical region of recipient.

† 1.0 mg/kg of piperoxan inj. into recipient's peripheral circulation.

‡ Mean arterial blood pressure $\left(\frac{\text{systolic} + \text{diastolic}}{2} \right)$.

§ Not recorded.

IA = Administration *via* arterial inflow to recipient's head. IV-R = Administration *via* recipient's femoral vein.

caudad vertebrae and the transverse processes of each of the 2 respective vertebrae, was then performed either between C-2 and C-3 or between C-3 and C-4. A suitable length of 21-gauge stainless steel wire was inserted lengthwise through a soft rubber sponge (0.75 by 5 by 0.25 cm), until the wire protruded through the sponge. The wire and sponge were pulled under the spinal cord, then the wire was placed in such manner that when brought to the ventral surface between the carotid arteries, it fell into correct position in the intervetebral spaces and when tightened occluded the vertebral venous sinuses and vertebral arteries. Circulation was established between the left common carotid artery of the anesthetized donor dog and the 2 common carotid arteries of the recipient,

and the 2 jugular veins of the recipient and left jugular vein of the donor. Two to 4 ml of heparin (1,000 μ /ml) were administered I. V. to the donor. Blood pressure was recorded from a femoral artery of each dog via a Statham transducer (P23AC) onto a Grass polygraph. Ten microcuries of I^{131} were administered into the arterial circulation to the recipient's head after thyroid glands of both animals had been saturated with iodine utilizing 0.25 ml of Lugol's solution, I. A., to the recipient's head. Circulatory leakage between head and body of the recipient was determined periodically by obtaining 3 ml blood samples from a femoral vein of the donor and recipient and determining amount of radioactivity per minute in each sample. There was absolutely no leakage observed in the 6

preparations utilized. Synthetic Angiotensin II[†] was administered either into the arterial inflow to the recipient's head or via the femoral vein to the recipient's peripheral circulation.

Results. The effects of Angiotensin II on donor and recipient blood pressures in the dog cross circulation preparation are summarized in Table I. Angiotensin II administered into the arterial inflow to the recipient's head in doses varying from 0.2 to 4 $\mu\text{g}/\text{kg}$ produced consistent pressor responses in both recipient and donor animals with pressure increments ranging between 12% and 50% above normal levels in recipient and 30% to 100% above pre-drug level in donor. Central hypertensive response was shorter in duration (0.5 to 5.0 min.) than pressor response in the donor animal (2.0 to 5.0 min.). Intravenous administration of Angiotensin II into the peripheral circulation of the recipient produced marked hypertensive responses of relatively long duration (2.5 to 6.0 min.), in the recipient only. In 2 preparations, 2 doses of Angiotensin II, 1 $\mu\text{g}/\text{kg}$, were injected into the head of the recipient and the hypertensive effect in both animals recorded. Subsequently, 1.0 mg/kg of piperoxan was slowly infused into the recipient's femoral vein. Angiotensin, 1 $\mu\text{g}/\text{kg}$, was then administered into the arterial inflow to the recipient's head and produced pressor responses in the donor only.

[†] Kindly supplied as Hypertensin by Ciba Pharmaceutical Products, Inc., Summit, N. J.

Intravenous administration of Angiotensin into the peripheral circulation of the recipient produced the usual pressor response. Vasopressin (0.1 $\mu\text{g}/\text{kg}$) administered *via* the arterial inflow to the untreated recipient's head produced hypertensive effects in the donor animal only.

Summary. Angiotensin II appears to produce an increase in blood pressure by 2 mechanisms: 1. A direct peripheral action on the vascular smooth musculature producing a marked increase in peripheral resistance, which is not blocked by piperoxan. 2. A central hypertensive effect, probably due to stimulation of central sympathetic structures and evoking peripheral sympathetic discharges, which are blocked by administration of a sympatholytic agent into the peripheral circulation.

1. Goldblatt, H., Lynch, J., Hanzal, R. F., Summerville, W. W., *J. Exp. Med.*, 1934, v59, 347.
2. Braun-Menendez, E., Page, I. H., *Science*, 1959, v127, 242.
3. Braun-Menendez, E., Paladini, A. C., *Circulation*, 1958, v17, 668.
4. Bumpus, F. M., Schwarz, H., Page, I. H., *ibid.*, 1958, v17, 664.
5. ———, *Science*, 1957, v125, 886.
6. Bianchi, A., DeSchaepdryver, A. F., Vleeschouwer, G. R., Preziosi, P., *Arch. Internat. Pharmacodyn. Therap.*, 1960, v124, 21.
7. Taylor, R. E., Page, I. H., *Circulation*, 1951, v4, 563.

Received November 21, 1960. P.S.E.B.M., 1961, v106.

Production of Streptolysin S by Streptococci Before and After Mouse Passage.* (26493)

IRVIN S. SNYDER[†] AND TOM R. HAMILTON

Department of Medical Microbiology, University of Kansas Medical Center, Kansas City, Kansas

No difference was found by Stollerman and Bernheimer (1) in streptolysin S production by strains of group A beta hemolytic streptococci isolated from patients with streptococcal pharyngitis or with rheumatic fever. That streptolysin S was produced in presence of yeast nucleic acid by strains of Lance-

field's group A as well as by certain strains of groups D, E, G, H, and L was reported by

* This investigation was carried out during the tenure of a Predoctoral Fellowship from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

[†] Present address: Dept. of Bacteriology, State University of Iowa, Iowa City.

Bernheimer(2).

The experiments reported here were performed (a) to determine quantitatively the amount of streptolysin S produced by streptococci isolated from clinical sources and grown in presence of yeast nucleic acid and (b) to study the effect of animal passage on level of production of this hemolysin by streptococci.

Materials and methods. A. Preparation of Yeast Nucleic Acid Medium. The medium utilized for growth of streptococci was prepared according to the method of Bernheimer and Rodbart(3). Beef heart for infusion (Difco) was substituted for the fresh beef heart. One ml of a Seitz-filtered solution of yeast ribonucleate (70 mg/ml) adjusted to pH 8.0 was added to the medium after it was sterilized by autoclaving. Control tubes received one ml of distilled water adjusted to pH 8.0. Before inoculation of the medium, freshly neutralized sodium thioglycollate was added to give a final concentration of 0.01%.

Seventy-five strains of streptococci isolated from clinical sources were used. Broth cultures of these organisms were kept frozen at -70°C until needed. Heart infusion medium was inoculated with the streptococci and incubated at 37°C for 6 hours. The yeast nucleic acid medium was inoculated with 0.1 ml of this suspension. Heart infusion medium lacking yeast nucleic acid was also inoculated. After 16 hours incubation at 37°C , the cultures were centrifuged and supernatant fluids were assayed for content of streptolysin S.

B. Assay of Streptolysin S. Serial 2-fold dilutions of each of the culture supernates, in phosphate buffer, were prepared in triplicate. To each tube of the first set of dilutions containing 1 ml of the diluted supernate was added 0.5 ml of a phosphate buffer pH 7.0 and 0.5 ml of 2% suspension of rabbit red blood cells. Because streptolysin O is inhibited by cholesterol and streptolysin S is inhibited by lecithin, a second set of serially diluted culture received 0.5 ml of a suspension of lecithin (5 mg/ml), while the third set of tubes containing serially diluted supernatant fluids received 0.5 ml of a suspension of cholesterol (50 $\mu\text{g}/\text{ml}$) in the place of the phosphate buffer. This procedure enabled the in-

vestigators to ascertain whether the hemolysin was due to streptolysin O, S, or both. After incubation for 45 minutes at 37°C , the tubes which showed hemolysis were removed from the water bath and centrifuged. The supernatant fluids were decanted, diluted 1:6 and optical density was determined at a wavelength of 5500 Angstroms, using a Coleman Universal spectrophotometer. The 50% hemolytic endpoint was calculated by use of conversion factors established by Von Krogh (4).

The unit of streptolysin S, as used in these experiments, is defined as that amount of streptolysin S dissolved in one ml of phosphate buffer which will lyse 50% of the rabbit red blood cells in 0.5 ml of a 2% suspension.

C. Preparation of Lecithin and Cholesterol Suspensions. Ten ml of 95% ethyl alcohol were added to 0.3 g of ovolcithin (Nutritional Biochemical Corp.). Solution was effected by stirring with a glass rod. Fifty ml of phosphate buffered saline were added to give a final concentration of 5 mg per ml.

Five mg of cholesterol (Difco) were suspended in one ml of absolute ethyl alcohol. This suspension was slowly poured into 100 ml of boiling water which then was filtered through paper.

Procedure for Passage of Streptococci through Mice. Mice were inoculated intraperitoneally with 0.5 ml of an 18 to 24 hour culture of streptococci. Between each animal passage the organisms were isolated from the mouse spleen or heart's blood and grown in Todd-Hewitt broth (Difco) for 18 to 24 hours.

Streptococci from such broth cultures were passed to mice by intraperitoneal inoculation; aliquots were frozen at -70°C for later evaluation of virulence and of production of streptolysin S. Virulence of the streptococci for mice was measured by intraperitoneal inoculation of 0.5 ml of 10-fold dilutions of an 18 hour culture (prepared from the frozen broth culture) into groups of 6 mice. The mice were observed for 12 days and LD_{50} determined by the method of Reed and Muench(5). For assay of streptolysin S, 0.1 ml of each streptococcal broth culture was inoculated into nu-

TABLE I. Production of Streptolysin S According to Group of Streptococcus.

Group	No. of strains studied	No. strains producing streptolysin S	Streptolysin S production (units/ml)	
			Range	Mean
A	43	42	10-128	52
B	6	0	—	
C Human	4	4	2-32	18
Animal	7	3	3-5	2
D	4	0	—	
G	11	10	2-18	7.5

cleic acid medium and incubated for 18 hours.

Results. Addition of cholesterol to the tubes did not inhibit the hemolysin while the addition of lecithin completely prevented hemolysis of the rabbit red blood cells. This indicated that streptolysin O was not present in sufficient amounts to be detectable and that the observed hemolysis was due to streptolysin S. Partial inhibition by lecithin and/or cholesterol was not observed.

Amounts of streptolysin S produced by 75 strains of streptococci of Lancefield groups A, B, C, D, and G are given in Table I.

The group A streptococci produced more streptolysin S than any of the other groups. Only one strain of group A streptococci produced streptolysin S in absence of yeast ribonucleate and this production was only 1.5 units per ml of culture supernate. However, one strain of group A failed to produce streptolysin S even in presence of yeast RNA. None of the group B or group D strains produced the hemolysin. A considerable difference is also apparent in production of streptolysin S by human and animal strains of group C.

Because considerable difference was noted between the groups with respect to streptolysin S production and also between members of the same group, a second experiment was performed to determine the effect of animal passage or adaptation to the host on production of streptolysin S. These results are shown in Table II.

This experiment indicated that production of streptolysin S can be modified by animal passage. In all strains which produced streptolysin S before animal passage, with one ex-

ception, streptolysin S production was augmented by such passage. The animal strain of group C, which did not produce streptolysin S before animal passage, did produce a limited amount after 8 animal passages. However, production of streptolysin S by strains of group D was not demonstrated at any time.

No increase in virulence of the streptococci for mice was obtained by animal passage.

Discussion. The significance of the production of streptolysin S by streptococci of Lancefield group A, C and G is not understood. However, some importance may be attached to the fact that most streptococcal infections in man are caused by members of these groups. It is similarly of considerable interest that streptolysin O(6) and streptokinase(7) also are produced only by members of these groups. That the largest amounts of streptolysin S are produced by streptococci of group A suggests that this streptolysin may have a significant role in human infections. The fact that this hemolytic toxin (S) is serum extractable has appealed to investigators relative to possible wide diffusion in tissues.

Leedom and Barkulis(8) showed that the enhanced virulence of streptococci after mouse passage was associated with decreased streptolysin S production. Although no enhancement of virulence of streptococci of groups A and C was obtained in our experiments after 8 to 10 mouse passages, the ability of the organism, with one exception, to produce streptolysin S was enhanced. However, these results do not preclude the possibility that the production of substances im-

TABLE II. Effect of Mouse Passage on Production of Streptolysin S by Strains of Streptococci of Groups A, C, and D.

Lancefield Group	Mouse passage level					
	0	2	4	6	8	10
A	10*	22	26	32	32	72
A	16	16	24	32	64	—
A	1	6	8	16	16	32
A	128	128	100	75	64	64
C	<1	5	5	6	8	10
D	<1	<1	<1	<1	<1	<1
D	<1	<1	<1	<1	<1	<1

* Units of streptolysin S/ml of culture supernate.

portant in the virulence of streptococci for mice may be at the expense of streptolysin S production and other products.

Summary. (1) Streptolysin S was produced by streptococci of Lancefield groups A, C and G. (2) Streptococci of group A produced considerably more streptolysin S than did groups C and G. (3) Strains of group C streptococci of human origin produced more streptolysin S than did animal strains of group C. (4) In general, the passage of beta hemolytic streptococci through mice enhanced the production of streptolysin S but did not enhance their virulence. Nonhemolytic strains were not affected by animal passage.

1. Stollerman, G. H., Bernheimer, A. W., *J. Clin. Invest.*, 1950, v29, 1147.
2. Bernheimer, A. W., *Bact. Rev.*, 1948, v12, 195.
3. Bernheimer, A. W., Rodbart, M., *J. Exp. Med.*, 1948, v88, 149.
4. In *Experimental Immunochemistry*, R. A. Kabat, and M. M. Mayer, eds., Charles C. Thomas, Springfield, Ill., 1948, p97.
5. In *Viral and Rickettsial Infections of Man*, T. M. Rivers, Ed., J. B. Lippincott, Philadelphia, Pa., 1952, 2nd edition, p72.
6. Todd, E. W., *J. Hyg.*, 1939, v39, 1.
7. Tillett, W. S., *Bact. Rev.*, 1938, v2, 161.
8. Leedom, J. M., Barkulis, S. S., *J. Bact.*, 1959, v78, 687.

Received November 28, 1960. P.S.E.B.M., 1961, v106.

Phosphoglucumutase Activity in Skeletal Muscles of Vitamin E-Deficient Chicks.* (26494)

WILLIAM R. HAZZARD† AND SAMUEL L. LEONARD

Dept. of Zoology, Cornell University, Ithaca, N. Y.

In a previous study concerning changes in enzymatic activity in abnormal skeletal muscle, it was shown that a decrease in phosphoglucumutase (PGM) activity occurred in this tissue in mice with hereditary muscular dystrophy(1) a finding which was similar to that reported in man with muscular dystrophy(2). For purposes of comparison, this report is concerned with measurements of PGM activity in red and white chicken muscle rendered dystrophic by maintenance of the birds on a vit. E-deficient diet. PGM activity was measured without addition of the coenzyme, glucose-1-6-diphosphate, and with it in order to determine maximal enzymatic activity present.

Methods. Ten male white Plymouth Rock chickens were fed *ad lib* from hatching on a vit. E-deficient diet similar to one reported (3) except that the protein consisted of 10% *Torula* yeast (deficient in selenium), 5% ca-

sein and 5% isolated soy protein supplemented by selenium (0.1 mg/kg of diet).† To the diet of 5 of these birds was added 80 mg α -tocopheryl acetate/kg. Autopsy under Nembutal anesthesia was performed at 5 weeks of age. Samples of white (breast) and red (leg) muscles were frozen with dry ice immediately after excision and stored at -20° until assayed.

PGM activity was determined by the method of Najjar(4) as refined by Bodansky(5). Aqueous homogenates of muscle were prepared in the cold and adjusted to 7.5 mg white muscle/ml and 15 mg red muscle/ml. To the reaction tubes was added the following in 0.1 ml volumes; 5×10^{-2} M glucose-1- PO_4 ; 8×10^{-2} M cysteine, freshly prepared and adjusted to pH 7.5; 1.2×10^{-2} M Mg SO_4 ; 4×10^{-1} M Tris buffer, pH 7.5; and 0.4 ml of either water or coenzyme (prepared as described below) for maximum PGM activity. The reaction was started by adding 0.2 ml of muscle homogenate freshly pre-

* Aided by grants from Muscular Dystrophy Assn. of America and by the Sage and Sackett Research funds, Dept. of Zoology.

† Medical student fellow, National Science Foundation.

‡ Chickens were raised and their diet devised and supervised by Corlette C. Calvert, Dept. of Poultry Husbandry, Cornell Univ.

TABLE I. PGM Activity in Vit. E-deficient Chick Skeletal Muscle.

Exp. (10 chicks)	Without coenzyme		With coenzyme	
	Fresh tissue*	NCPN†	Fresh tissue*	NCPN†
White muscle				
Vit. E-deficient	26 \pm 4.0	7.1 \pm 1.4	66 \pm 4.8	18 \pm 1.9
Control	47 \pm 1.3	13.6 \pm .7	97 \pm 3.0	28 \pm 1.7
P value	<.01	<.01	<.001	<.01
Red muscle				
Vit. E-deficient	7.8 \pm 1.2	2.7 \pm .3	27 \pm 2.1	9.3 \pm .4
Control	9.1 \pm 1.0	3.0 \pm .4	29 \pm 1.1	9.7 \pm .7

* mg P/100 mg tissue/hr \pm S.E.† mg P/mg non-collagenous protein nitrogen/hr \pm S.E.

pared. After 4 minutes of incubation at 37.5°, the reaction was stopped by adding 1 ml of 5 N H₂SO₄. The mixture was diluted to 5 ml, heated for 7 minutes at 100°, filtered and 1 ml aliquot taken for P determination(6). The results are reported as mg glucose-6-PO₄ P formed/100 mg fresh tissue/hr. Non-collagenous protein nitrogen (NCPN) of the muscle was determined(7) and enzyme activity reported also on this basis.

Although pure α -D-glucose 1-6-diphosphate (coenzyme) is not available to permit saturation of the reaction mixture with coenzyme to determine maximum PGM activity, nevertheless it is possible to estimate maximum activity, as previously demonstrated in mouse muscle(1), by adding increasing amounts of a boiled muscle extract known to contain coenzyme, when prepared according to Cardini *et al.*(8), to a series of PGM reaction mixtures. By plotting the reciprocals of the concentration of added coenzyme (mg boiled muscle extract) *vs* velocity of the reaction (mg P/100 mg tissue/hr), a linear relationship obtains and extrapolation to infinite coenzyme concentration permits estimation of maximum PGM activity. In the present experiment, boiled extracts containing coenzymes were prepared from normal white chick muscles and added in amounts equivalent to 2-16 mg tissue in 0.4 ml volume. Linear increases in PGM activity occurred with addition of 2-8 mg equivalents of boiled extract but addition of more than 8 mg up to 16 mg failed to increase PGM activity further. Thus, a direct determination of maximum PGM activity was possible by adding 8-16 mg of the white muscle extract in these ex-

periments which thereby increased the technical efficiency of the assay.

Results and discussion. The muscular lesions developed in the vit. E-deficient chicks varied from moderate to severe although the experimental and control birds consumed equal amounts of their diet and had similar average weight at autopsy (529 *vs* 534 g respectively). Grossly, white muscles exhibited more severe lesions than the red.

Results in Table I show that PGM activity in white muscle from vit. E-deficient chickens decreased by 48% when measured without added coenzyme and 36% with maximum coenzyme present, based on NCPN content of the tissues. In most instances, the decline in PGM paralleled the severity of the lesions as noted by gross inspection at autopsy. The red muscle, in contrast, failed to show a significant alteration in PGM activity. The activity of normal white muscle was 3-4 times that of red muscle when assayed either with or without maximum coenzyme additions.

The percent decline in PGM activity (calculated on the basis of NCPN and with added coenzyme) in white muscle of deficient chicks at 5 weeks of age is similar to that of dystrophic mouse muscle(1) at 13 weeks of age (36% *vs.* 39% for the latter). Ratios between PGM activity of vit. E-deficient and control chicken white muscles, when calculated from determinations made in absence and presence of added coenzyme are not markedly different (.52 *vs.* .64), thus permitting the inference that coenzyme content of the muscle differs by only a little more than does the PGM. In contrast, these ratios in the dystrophic mouse were .32 without

added coenzyme and .61 with coenzyme, from which it was inferred that a great loss in coenzyme accompanied the lesions in the mouse (1). Thus it appears that loss of coenzyme in the dystrophic chick muscle is proportionately not as large as in the dystrophic mouse muscle. Normal white chick muscle contains more G-1-6-diphosphate than rat or mouse muscle. Repeated experiments showed that saturation of the PGM system with this coenzyme was not possible with additions equivalent to 8-16 mg of boiled extract prepared from rodent muscle whereas saturation was readily achieved when white chick muscle was employed.

Phosphorylase activity, as well as PGM, is considerably greater in white than in red muscle of the normal chick(3). It is only in white muscle that phosphorylase is decreased in the vit. E-deficient chick. The sensitivity of white muscle to the vitamin deficiency might be correlated with the initially high levels of phosphorylase and PGM, and a longer period of feeding the deficient diet might possibly affect the red muscle as well. PGM activity in the back muscles of rabbits fed a vit. D-deficient diet was lower than normal but the results were not statistically significant(9).

Summary. Phosphoglucumutase activity determined with and without added coenzyme

(glucose-1-6-diphosphate) was increased in white muscles of chicks fed a vit. E-deficient diet, but was unaffected in red muscle. Muscle lesions were more extensive in the white muscle. Normally white muscle contains more PGM than red muscle. The coenzyme for PGM is much more plentiful in white chick muscle than in rat or mouse muscle so that direct determination of maximum PGM activity by saturation of the system with added coenzyme is possible, thus eliminating the necessity of extrapolating to infinite coenzyme concentration.

1. Hazzard, W. R., Leonard, S. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v102, 720.
2. Dreyfus, J. C., Schapira, G., *Compt. rend. Soc. biol.*, 1954, v147, 1145.
3. Nesheim, M. C., Leonard, S. L., Scott, M. L., *J. Nutrition*, 1959, v68, 359.
4. Najjar, V. A., *J. Biol. Chem.*, 1948, v175, 281.
5. Bodansky, O., *ibid.*, 1958, v232, 859.
6. Fiske, C. H., Subbarow, Y., *ibid.*, 1925, v66, 375.
7. Lilienthal, J. L., Jr., Zierler, K. L., Folk, B. P., Buka, R., Riley, M. L., *ibid.*, 1950, v182, 501.
8. Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F., Trucco, R. E., *Arch. Biochem.*, 1949, v22, 87.
9. Carpenter, M., McCay, P., Caputto, R., *PROC. SOC. EXP. BIOL. AND MED.*, 1958, v97, 205.

Received December 19, 1960. P.S.E.B.M., 1961, v106.

Estrogen Therapy in Immature Female Rats with Posterior Hypothalamic Lesions. (26495)

ALAN CORBIN AND B. A. SCHOTTELIUS

Dept. of Physiology, College of Medicine, State University of Iowa, Iowa City

Recent findings(1-5) support the concept of hypothalamic control of the hypophyseal-gonad axis. Corbin and Schottelius(6) have further implied a dual capacity of the hypothalamus, as regards puberal initiation and sexual maturation. Failure of the puberty-parturition continuum may be attributed to ovarian hypofunction, representing a secondary effect of posterior hypothalamic damage.

Gale(7) tested the effectiveness of replacement therapy in hypothalamically lesioned,

pregnant rats, by administering progesterone and estradiol benzoate and preventing gestational impairment and abortion. Information is relatively scarce, however, relating estrogen therapy to reproductive organ maturation in immature female rats with discrete hypothalamic lesions. The response of the reproductive organs to estrogen replacement in prepuberal female rats with posterior hypothalamic lesions is the object of this study.

TABLE I. Means and Standard Errors of Body Weights, Hypophyses, Reproductive Organs, Vaginal Opening and Cycling in Normal, Sham-Operated, Lesioned Untreated, Lesioned Estrogen-Treated, and Lesioned Oil-Treated Female Albino Rats. No. of animals per group in parentheses.

	Body wt (g) (21 days)	Body wt (g) (75 days)	Hypophy- sis wt (mg) (75 days)	Reproduc- tive organ wt (mg) (75 days)	Day vagina opened	Cycling be- gan, days post vaginal opening	Vagina opened days post treatment	Cycling be- gan, days post vaginal opening
Group 1, normal control (13)	39.1 ± 2.6	178.3 ± 3.7	6.41 ± .51	450.7 ± 10.4	37 ± .51	7 ± .25		
Group 2, sham-operated control (11)	41.6 ± 2.3	170.8 ± 5.7	6.60 ± .46	490.6 ± 29.1	36 ± .54	7 ± .25		
Group 3, lesioned untreated control (8)	42.5 ± 1.0	142.8 ± 10.3	2.70 ± .20	173.0 ± 23.0	60 ± 5.7	Diestrus		
Group 4, lesioned estrogen-treated (8)	42.0 ± 1.2	152.3 ± 18.1	3.60 ± .27	443.2 ± 24.7	45 (2 animals)	"	53 ± 1.9 (6 animals)	Constant estrus
Group 5, lesioned oil-treated control (4)	42.0 ± .84	144.5 ± 12.9	3.60 ± .49	166.6 ± 30.1			69 ± 2.9	Diestrus

Methods and materials. Forty-four, 21-day-old female albino rats (Holtzman) were divided into the following groups: Group 1 (13 animals) was the normal control group; Group 2 (11 animals) was the sham-operated control group; Group 3 (8 animals) consisted of the lesioned, untreated subjects; Group 4 (8 animals) contained the lesioned, estrogen-treated rats, and Group 5 (4 animals) represented the lesioned, oil-treated control group. Bilateral destruction of the mammillary body (MB) region was performed on day 21. Lesioning technic was a modification of that described by Corbin and Schottelius (6). Animals were allowed Rockland rat diet and water *ad lib*. Body weights were recorded every 5 days. From the time of vaginal opening, smears were examined daily up to time of autopsy to determine presence and length of cycles. On day 52, Group 4 received 0.5 μ g estradiol valerate in 0.1 ml of sesame oil, I.M. (a repository dose, effective for approximately 3 weeks), and Group 5 received 0.1 ml of the vehicle. All rats were observed for behavioral changes, and were sacrificed on day 75. Fresh weights of pituitaries and reproductive organs (total weight of ovaries + oviducts + uteri + vagina) were recorded. Brains were fixed in 10% formalin for 5 days, sectioned at 10 μ and stained in H and E. Data were statistically analyzed by Students' "t" test. A probability of ≤ 0.05 was considered to be significant.

Results. Results are summarized in Tables I and II. Histological examination of lesioned brains revealed damage limited to areas at the level of the MB. Animals with posterior hypothalamic damage showed definite inhibition of reproductive organ development, their organs being less than $\frac{1}{2}$ the weight of the normal controls. Groups 3 and 5 had the smallest reproductive organ and pituitary weights. These animals exhibited thin, unvascularized, atrophied sex organs. Moreover, the ovaries were pale, showing retarded follicular and luteal development. Pituitaries of animals in Groups 3, 4, and 5 were significantly smaller than those of the normal and sham-operated controls, indicating a direct correlation between hypophyseal

TABLE II. Table of "t" Values for Normal, Sham-Operated, Lesioned Untreated, Lesioned Estrogen-Treated and Lesioned Oil-Treated Female Albino Rats at 75 Days.

Groups compared	Body wt	Hypophysis wt	Reproductive organ wt	Day of vaginal opening
1 & 2	1.10	.28	1.33	1.35
1 & 3	3.25*	4.56*	11.0 *	4.03*
1 & 4	1.42	4.0 *	.24	
1 & 5	2.52†	4.18*	8.90*	10.3 *
2 & 3	3.58*	3.60*	8.27*	4.21*
2 & 4	.97	5.80*	1.24	
2 & 5	1.37	4.50*	7.76*	10.8 *
3 & 4	.43	2.72†	8.31*	1.16
3 & 5	.10	1.90	.17	1.40
4 & 5	.33		7.13*	4.57*
4 & 3 + 5				2.25‡

* $P \leq .01$

† $P \leq .02$

‡ $P \leq .05$

weight and hypothalamic integrity.

Vaginae of Groups 1 and 2 opened between days 36 and 37, with a cycling pattern being established 7 days later. In contrast, Groups 3 and 5 (which are theoretically identical) did not exhibit vaginal opening until days 60 and 69, respectively, and thereafter gave continuous leucocytes smears. Thus, sesame oil alone had no effect upon Group 5. Group 4 included 2 animals whose vaginae opened on day 45 which, nevertheless, remained in constant diestrus.

The vaginae of the six estrogen-treated rats of Group 4 opened on day 53, one day after treatment. The reproductive organs of Group 4 were equivalent in weight to those of the normal and sham-operated controls, at least by the time of sacrifice.

While cannibalism and viciousness were present in some lesioned subjects, there were no indications of hyperphagic behavior or obesity. Estrogen was ineffective in relieving the hostile attitude of such animals. Although a significant difference existed between body weight of the normal controls and the lesioned untreated animals, this may be attributed to the fact that the lesioned animals reduced their food intake for about 2 weeks after operation.

Discussion. Comparison of the reproductive organs of a normal control, sham-operated control, lesioned untreated, lesioned estrogen-treated and lesioned oil-treated rat is shown in Fig. 1.

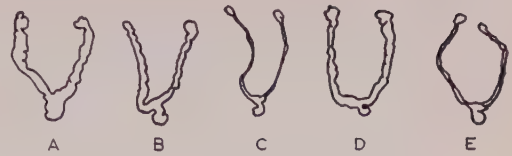


FIG. 1. Comparison of reproductive organs of normal control (A), sham-operated control (B), lesioned untreated (C), lesioned estrogen-treated (D) and lesioned oil-treated (E) rat at 75 days. Redrawn from photograph.

Ovarian failure in the lesioned rats not receiving estrogen therapy is evidenced by delayed puberty, reproductive organ immaturity and persistent diestrus state. Pituitary weights are compatible with such gonadal hypofunction; but, as is generally conceded, weight alone is not a reliable index of hypophysial secretory function. It appears evident that posterior hypothalamic damage prevents elaboration or release of hypophysial gonadotropins, thus inhibiting ovarian estrogen secretion; hence, the atrophy of the reproductive complex.

Those animals receiving estradiol valerate showed vaginal opening within one day of injection, and exhibited reproductive organ growth comparable in weight to those of normal and sham-operated controls, and in considerable excess of Groups 3 and 5. The hormone treated animals lacked developing ova; they exhibited a constant estrous state, as evidenced by continuous cornified smears. Absence of estrual cycling in these animals strengthens the concept of the dependence of gonadotropin release upon hypothalamic function. The lack of ova production and the state of impaired estrual cycling in all the lesioned animals (indicating absence of hypophyseal-ovarian feedback) strongly imply failure of the FSH-LH mechanism whose maintenance is, in turn, dependent upon normal hypothalamic integrity(8-11).

Statistically, Groups 3 and 4 did not show a significant difference in day of vaginal opening which is misleading due to the fact that the opening was induced by estrogen treatment. Since the day of vaginal opening in Groups 4 and 5 is significantly different, and since Groups 3 and 5 are essentially identical, the latter groups were combined for statistical analysis. Consequently, upon com-

parison with Group 4, a significant difference was seen in day of vaginal opening.

Summary. Twenty, 21-day-old female albino rats were subjected to bilateral lesions of the region of the mammillary bodies. Within one day of receiving a repository dose of 0.5 μ g of estradiol valerate, all 6 recipients showed vaginal opening with subsequent constant estrus and reproductive organ growth. Four animals receiving sesame oil and 8 receiving no treatment exhibited definite signs of delayed puberty and reproductive organ immaturity. These results indicate the inability of the prepuberal hypothalamus to elaborate or secrete those neurosecretory substances essential for the functional sexual state of the mammal, and the dependence of estrogen secretion upon normal hypothalamic activity as mediated *via* the hypothysis.

1. Greer, M. A., *Rec. Prog. Horm. Res.*, 1957, v13, 67.

2. Bogdanove, E., Schoen, H., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v100, 664.

3. Donovan, B. T., van der Werff ten Bosch, J. J., *J. Physiol.*, 1959, v147, 78.

4. Gellert, R. J., Ganong, W. F., *Acta Endocrin.*, 1960, v33, 569.

5. Cook, A. R., *Texas Reports on Biol. and Med.*, 1959, v17, 512.

6. Ccerbin, A., Schottelius, B. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v103, 208.

7. Gale, C., McCann, S. M., *The Physiologist*, 1959, v2, 43.

8. Benoit, J., Assenmacher, I., *J. Physiol. (Paris)*, 1955, v47, 427.

9. Moszkowska, A., *C. R. Soc. Biol.*, 1959, v153, 1945.

10. Sawyer, C. H., *Rec. Prog. Endocrinology of Reproduction*, Acad. Press, N. Y., 1959, 1.

11. Segal, S. J., Johnson, D. C., *Arch. Anat. Micr. Morph. Exp.*, 1959, v48, 261.

Received December 20, 1960. P.S.E.B.M., 1961, v106.

Adaptation of Fluorescent-Microscopy to Determination of Genetic Variations in Mouse Mammary Tumor Agent. (26496)

ERIC R. BROWN* AND JOHN J. BITTNER

*Division of Cancer Biology, Department of Pathology, Medical School, University of Minnesota
Minneapolis*

It was previously found that fluorescent microscopy could be adapted for studying the antigenic mosaic make-up of tissues containing the mouse mammary tumor agent (MTA) (1). While fluorescent microscopy, as Hughes(2) indicated, has definite limitations in differentiating neoplastic from normal tissues, we have found that this method offers certain advantages over the routine serologic and neutralization tests used for such studies.

"Incidence" and "age of occurrence" of mammary tumors in the mouse, have been shown to be influenced by certain inherited factors(3). Especially implicated have been the hormonal make-up of the animal and the

genes responsible for natural protection or susceptibility to the MTA. Certain inbred stocks of mice have been developed that not only have a high incidence of tumors at an early age, but can pass the agent to succeeding generations by nursing. Inbred genetically pure mice have also been selected which are highly resistant to the MTA.

Little is known about the antigenic changes occurring within the MTA when it is passed from one strain of mice to another. In part, this is due to inability to isolate and purify a single entity which might be termed the "agent" and in part due to the time period necessary for neutralization tests. Inhibition or neutralization tests are the only reliable method so far available for determining the effect of antisera on the MTA. Unfortunately, it requires a period of 6 months, in

* Post-doctoral Fellow of Am. Cancer Soc., Inc. This work was supported in part by grants from Am. Cancer Soc., Minnesota Division, U.S.P.H.S., and Am. Cancer Soc., Inc.

the case of positive tests, to 2 years to obtain the results of such studies. Despite these handicaps, some antigenic variations are known to occur when the MTA is transferred to different strains: Bittner and Imagawa (4) described the results of neutralization tests using antisera prepared from MTA extracts. They noticed that antigenic differences were apparent between the mammary tumor agent of a cancerous stock and that of F_1 hybrids having mothers of the same strain and that the antigenic properties of the agent in a transplanted tumor may become altered with continued passage of the tumor in agent-free animals. In collaboration with Hirsch and others, it has been reported(5) that antigenic variations occurred in the MTA depending upon passage of the tumors and the hosts. It was found that antisera produced against the agent in tumors transplanted in mice of the inbred strain neutralized the MTA in extracts of the same tumor grown in either inbred or agent-free F_1 animals. On the other hand, antisera elicited against the tumor which had been transplanted in F_1 hybrids neutralized the agent from similar tumors but did not neutralize the agent in the same tumors carried in inbred animals of the strain of origin. These experimental data could indicate that the antigenic characteristics of the MTA had been influenced by incorporation of normal tissue component(s) from the host.

Technic. To expand the initial findings on the antigenic changes mentioned above it was felt that fluorescent microscopy would offer several advantages: a) microscopic visualization of site of reactivity on the antigen, b) more rapid method for obtaining results and c) a greater specificity in the reaction.

Rabbit or guinea pig antitumor sera were prepared, as previously reported(1), against the Z tumor in Z mice and the A tumor in A mice where a minimum of 3 rabbits or 12 guinea pigs was used for each serum preparation. After exsanguination the sera were pooled and adsorbed with various normal mouse tissues to remove common antibodies. Ammonium sulfate fractionations were run to obtain a higher concentration of gamma globulins and the material was then conju-

gated with fluorescent dye(s) prior to testing against tumors derived from mice of different strains.

The preparation of isothiocyanate for conjugation to the antisera was carried out by the method of Riggs(6). This dye was found to give maximum contrast when viewed under ultra-violet light. In addition to the use of isothiocyanate alone, the combination technic of Lissamine-Rhodamine RB 200 conjugated to a 1% albumin solution and then mixed (1:20) with the isothiocyanate conjugated antisera, was found effective in eliminating non-specific background fluorescence. Preparation of the Lissamine-Rhodamine RB 200 was undertaken by the technic of Smith and associates(7). Method of preparation and staining of tissue slides was essentially that of Cherry and colleagues(8) using fluorescent-free paraffin mounts and tissues of 3 μ or less in thickness. The material to be studied was fixed in 10% formalin for 24 hours, carried through the usual dehydration cycles, cut, stained with the conjugated antisera, washed with phosphate buffered saline, and mounted under a coverslip with buffered glycerol.

The tissues found most convenient for adsorbing out common or normal tissue antibodies was liver powder; method of preparation was that of Coons and associates(9). Mouse liver powder was obtained from both the strain C and strain Ax mice which did not contain the MTA. The rabbit or guinea pig antisera prepared against the Z tumor in Z mice, and the A tumor in A mice, were then adsorbed with one or the other of these liver powders prior to conjugation with the fluorescent dyes. The treated antisera were tested for specificity and degree of fluorescence against host tumor specimens by the immuno-fluorescent technic described above. Normal mouse tissues, a spontaneously occurring sarcoma, and an adenocarcinoma produced in an agent-free animal were used for controlled antigen studies. Normal guinea pig or rabbit sera were conjugated with the fluorescent dyes and used to control the serum preparations. For each slide stained with a fluorescent dye the next tissue slice from the microtome was stained with hemo-

toxylin and eosin for routine histologic comparison.

To avoid erroneous interpretation of the material and to have a more unbiased appraisal of the technic, "unknown" tumor samples were submitted for evaluation. One of the authors (JJB) selected animals bearing a tumor derived from a known strain and whose history was well pedigreed, as an unknown for the other author to evaluate. When received, the "unknown" tumor was numbered and prepared for fluorescent microscopy in the manner described above. "Unknowns" were compared to known tumors and degrees of fluorescence found using the various tumor antisera was noted. When the experiment was completed the findings were evaluated for specificity.

Results. Early in these studies it was found that while either the rabbit or the guinea pig would respond to the antigenic stimuli of the mouse tumor antigen, that considerable "normal tissue antibodies" were also elicited by the animal. Most of the normal tissue antibodies were removed by adsorption (4 days with 6 hour changes of adsorbing tissue) with normal mouse liver powder and only a small amount of fluorescence in the preparations was then due to normal tissue antibodies. Despite the use of adsorbing powders a slight degree of nonspecific fluorescence was apparent in all of the studies. Best results were obtained when strain C liver powder was used against the anti-Z or anti-A tumor sera to eliminate nonspecific fluorescence. Despite the handicap of a certain amount of non-specific fluorescence, valid results could be obtained. Differences could be denoted in degree of fluorescence when the various antisera were reacted against the tumor material. The results (Table I) agree quite well with the neutralization studies reported by Bittner and associates(5), and indicate that this technic allows a faster, more specific method for studying degree of specificity and reactivity of anti-MTA sera when reacted on heterologous tumors.

Discussion. Fluorescent-antibody studies on antigenic variations of the MTA when it is passed in various host animals offer a rapid, inexpensive, and accurate technic for

determining specificity of the reaction. The only reliable method for determining antigenic variations in the MTA from normal tissues is the bio-assay method. Investigations are now in progress to combine this method with the fluorescent antibody technic. Evaluation of results of these experiments will require at least 2 years. Studies are now underway to investigate antigenic changes which take place in MTA when it is carried in normal mouse tissues.

The fluorescent-antibody technic has definite limitations in defining the antigenic mosaics of the mouse mammary tumor agent. The method is limited by the quality, specificity and reactivity of the antisera under investigation. Considerable skill is necessary to distinguish between the natural fluorescence or non-specific fluorescence of the tumor preparation and that arising as a result of specific protein-protein interactions. In not every case is it possible to avoid these pit-falls. Despite these drawbacks, from Table I it becomes apparent that the fluorescent-antibody technic has a high degree of accuracy, and in particular is a method which reduces the time factor for such studies from 2 years to a few days. The conjugated antisera can be stored indefinitely by rapid freezing without appreciably changing the reactivity of the preparation, thus it is possible to develop a battery of conjugated antisera specific for each of the various materials associated with the MTA. Use of the immunofluorescent technic allows a method to be developed for better understanding of the genetic relationships of MTA and tumors containing MTA when correlated with animal studies.

Summary. A fluorescent-antibody system is reported which allows rapid analysis of the minor antigenic differences between mouse mammary tumor agent (MTA) extracts obtained from different sources. This method correlates very well with the bio-assay method reported by Bittner and associates. An analysis of the antigenic component can be made within a few hours after receiving the MTA extract. In the older bio-assay method up to 2 years of observations were necessary for proper evaluation. The disad-

TABLE I. Fluorescent-Antibody Studies on Mouse Tumor Agent (MTA). Summary of effects of rabbit anti-MTA sera adsorbed with normal C strain mouse liver powder, fractionated, and conjugated with isothiocyanate, then reacted against MTA derived from transplanted tumors. Tumors were transplanted in either inbred or hybrid mice for 7 passages.

Conjugated rabbit antisera preparation	A tumor in				Z tumor in				Normal mouse tissue, no MTA
	A mice	Z mice	AxZbFl mice	AxZbFl/ZbAxFl mice	A mice	Z mice	AxZbFl mice	AxZbFl/ZbAxFl mice	
Anti-A sera adsorbed with strain C liver powder	4+	NT	2+	3+	NT	±	1+	2+	±
Anti-Z sera adsorbed with strain C liver powder	±	NT	1+	2+	NT	4+	2+	2+	±
Anti-A sera fractionated and adsorbed with strain C liver powder	4+	NT	1+	2+	NT	±	±-1+	1-2+	±
Anti-Z sera fractionated and adsorbed with strain C liver powder	±	NT	±	1+	NT	4+	3+	3+	±
Anti-A sera whole unadsorbed	4+	NT	3-4+	4+	NT	3-4+	4+	4+	3-4+
Anti-Z sera whole unadsorbed	3-4+	NT	3-4+	3+	NT	4+	4+	4+	4+
Normal control sera									
Normal rabbit sera whole, unadsorbed	1-2+	NT	1-2+	1-2+	NT	1+	1-2+	1-2+	1-2+
Normal rabbit sera adsorbed with strain C liver powder and fractionated	0	NT	±	0	NT	±	±	±	±

NT = no tumor take with transplanted tumor; 4+, maximum fluorescence; 3+, strong fluorescence; 2+, avg fluorescence, not too bright; 1+, some slight fluorescence; ±, very slight fluorescence; 0, no apparent fluorescence.

vantages of the fluorescent-antibody system include, a) interference by non-specific fluorescence, b) limitations imposed by antisera employed, c) removal of normal tissue antibodies, and d) selection of appropriate fluorescent dyes for conjugation to the sera. These disadvantages can be partially overcome by carefully selecting the antigens to be used for preparing antisera, adsorption of antisera with mouse liver powder extracts, concentration of globulin fractions prior to conjugation, and use of sharply contrasting fluorescent dyes.

1. Brown, E. R., Bittner, J. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1961, v106, 303.

2. Hughes, P. E., *Cancer Res.*, 1958, v98, 898.

3. Bittner, J. J., *Ann. N. Y. Acad. Sci.*, 1958, v71, 943.

4. Bittner, J. J., Imagawa, D. T., *Cancer Res.*, 1955, v15, 464.

5. Bittner, J. J., Hirsch, H. M., Ross, J. D., Gabrielson, R., *ibid.*, 1959, v19, 918.

6. Riggs, J. L., Masters Thesis, Univ. of Kansas, 1957.

7. Smith, C. W., Marshall, J. D., Jr., Eveland, W. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v102, 179.

8. Cherry, W. B., Goldman, M., Carski, T. R., Public Health Service Publication No. 729, U. S. Gov't. Printing Office, Washington, D.C., 1960.

9. Coons, A. H., *et al.*, *J. Exp. Med.*, 1955, v102, 49.

Received January 3, 1961. P.S.E.B.M., 1961, v106,

Effect of Oleyl Alcohol on Esterification of Cholesterol in a Pancreatic Extract. (26497)

PAUL W. O'CONNELL (Introduced by M. H. Kuizenga)

Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

In describing an enzyme in acetone powder of pancreas which esterifies vit. A, Pollard and Bieri(1) noted the similarities (nonspecificity of fatty acids and lack of requirements for coenzyme A activation) to cholesterol-esterifying enzymes of the pancreas(2,3). Employing a pancreatic extract with the same characteristics for study of cholesterol esterification, we have observed that addition of the lipid-soluble, unsaturated aliphatic alcohol, oleyl alcohol, resulted in its esterification and an increase in extent of sterol esterification.

Methods and materials. The standard reaction mixture was an emulsion of the fatty acid and cholesterol (one member of the substrate-pair being isotopically labeled) in 0.15 *M* potassium phosphate buffer, pH 6.2, containing sodium taurocholate (except where omission is noted in the tables), 10 mg per ml, and bovine serum albumin, 3 mg/ml. The fatty acid to cholesterol ratio was uniformly 2.5 to 1. The substrates were added to the buffered solution in a small volume of ether and the mixture was swirled in a warm water bath while a vacuum was applied to the flask.

Palmitic-1-C¹⁴ acid and cholesterol-4-C¹⁴ were purchased from Volk Radiochemicals and linoleic-1-C¹⁴ acid from Nuclear-Chicago. The unlabeled linoleic acid was obtained from Mann Fine Chemicals; palmitic acid was Eastman Kodak material. Commercial cholesterol was purified according to Hernandez and Chaikoff(3).

The enzymatic reaction was initiated by adding to 7 ml of the emulsion (equilibrated with shaking at 37.5°C) one ml of a pancreatic extract prepared by homogenizing fresh frozen pork pancreas in 5 volumes of cold 0.25 *M* sucrose (or, in some experiments, glycerol:water, 1:1) and centrifuging lightly. The fluid portion between a white surface layer and the centrifuged residue constituted the enzyme source. After a 15 min incubation,

the reaction mixture was extracted twice with 15 ml volumes of hot chloroform. Where the production of cholesterol esters was anticipated, 10 mg of cholesteryl linoleate or palmitate were added as a carrier. The pooled chloroform extracts were concentrated to dryness and the residue taken up in Skellysolve B. In a single experiment, to be described, extraction was made with chloroform:methanol, 2:1 and the extract was washed with dilute alkali before subsequent Dowex-1 treatment. In early experiments, unreacted acid was removed by washing the Skellysolve solution with aqueous ammoniacal ethanol. Later it was found more effective to pass a wet ether solution of the total lipid extract over Dowex-1 (OH⁻ cycle), using the procedure of Savary and Desnuelle(4). An aliquot of the solution after resin treatment could be counted for estimation of fatty acid incorporated into total neutral lipid.

The chromatographic separation, "Scheme A" or "Scheme B", of Hirsch and Ahrens(5), scaled to 4 g silicic acid (Bio-Rad Laboratories) was used for isolation of the cholesterol esters. Fractions were collected in 10 ml volumes in vials used for the Packard liquid scintillation spectrometer. The cholesterol esters, or simple esters, are eluted by one per cent ether in petroleum ether (Skellysolve B) and will be referred to as the ester fraction. Glyceride esters are eluted in subsequent fractions. After evaporation of the eluting solvents, a sample was dissolved in 10 ml of toluene containing 3 g 2,5-diphenyloxazole and 0.1 g 1,4-di[2-(5-phenyloxazolyl)]benzene per liter and radioactivity measured in the spectrometer.

The oleyl alcohol employed in initial experiments was commercial material (Archer-Daniels-Midland, "Adol 85") stated to be 80% oleyl alcohol and the remainder as shorter chain saturated alcohols. In most of the experiments to be described, a purified sample, at least 95% pure, provided by Dr.

TABLE I. Effect of Oleyl Alcohol on Palmitate- and Linoleate- C^{14} Incorporation into Esters by Pancreatic Extracts.

Fatty acid substrate	Oleyl alcohol added (μ moles/flask)	Radioactivity ester fraction (counts/min.)
Palmitate	—	725 805
Linoleate	—	8,150
Palmitate	14	33,000 35,000
Palmitate	1.4	3,175
Linoleate	30	15,770
"	3.0	8,650

Each flask contained 7 ml of an emulsion of fatty acid (palmitate, 5 μ moles/ml, or linoleate, 10 μ moles/ml, containing 4×10^3 counts/min. of respective C^{14} -labelled acid/ μ mole), and cholesterol as described in *Methods*. The enzyme was added in 1 ml of extract of pork pancreas in glycerol-water, 1:1. Incubation, 15 min. at 37.5°C.

L. W. Beck, Procter and Gamble Co., was used.

Results. Varying amounts of oleyl alcohol were added to the palmitic acid-cholesterol and linoleic acid-cholesterol reaction mixtures, respectively, just before introduction of the pancreatic extract. The radioactivity appearing in the isolated ester fraction is shown in Table I.

When an equimolar amount of oleyl alcohol replaced cholesterol in the emulsion, 11,000 cpm of palmitate were incorporated in the ester fraction during a 15 minute incubation, and 13,675 cpm in 30 minutes. A small amount of clear oil obtained from silicic acid column fractions corresponding to the peak of ester radioactivity had an infrared spec-

trum consistent with an ester containing a double bond, and formation of oleyl palmitate was thus inferred.

The same pancreatic extract was employed as the enzyme source for preparations in which the radioactive label was present in palmitic-1- C^{14} acid or cholesterol-4- C^{14} . The emulsions with radioactive cholesterol were prepared in presence and absence of sodium taurocholate. The results are summarized in Table II.

TABLE III. Effect of Sodium Taurocholate on Esterification of Palmitate- C^{14} with Oleyl Alcohol and Cholesterol, Alone or in Combination, by a Pancreatic Extract.

Alcoholic substrate	Sodium taurocholate (70 mg/flask)	Radioactivity (counts/min.)	
		Total neutral lipid	Ester fraction
Cholesterol	—	1,675	430
	+	2,825	2,000
Oleyl alcohol	—	3,125	2,215
	+	7,725	2,800
Cholesterol + oleyl alcohol	—	13,100	10,000
	+	22,625	18,225

Conditions were same as in Table II.

Table III contains additional information on the effects of taurocholate. The emulsions were prepared without the bile salt and with the usual concentrations of palmitate- C^{14} and either or both oleyl alcohol and cholesterol. Taurocholate was added to the appropriate flasks as an aqueous solution during the equilibration period. Extraction of these reactions was carried out with chloroform:methanol, 2:1.

TABLE II. Esterification of Palmitic Acid with Oleyl Alcohol and Cholesterol, Effect of Sodium Taurocholate.

Alcoholic substrate (μ moles/flask)		Radioactive substrate	Sodium taurocholate (70 mg/flask)	Radioactivity of ester fraction (counts/min.)
Cholesterol	Oleyl alcohol			
14	—	Palmitic acid*	+	345
—	14	" "	+	2,955
14	14	" "	+	9,460
14	—	Cholesterol†	—	35
14	—	"	+	335
14	14	"	—	55
14	14	"	+	2,640

* 4×10^3 counts/min./ μ mole.

† 5×10^3 counts/min./ μ mole.

Each flask contained 7 ml of an emulsion of palmitic acid (5 μ moles/ml) and appropriate alcoholic substrate as described in *Methods*. Enzyme was added in 1 ml of extract of pork pancreas in 0.25 M sucrose. Incubation, 15 min. at 37.5°C.

Discussion. The effects of additives on enzymatic reactions involving lipids must frequently be interpreted with consideration for the possibility that physical changes in the system have altered the availability of the substrate to the enzyme. In the present instance, several reasons support the idea that some interaction of the 2 alcoholic substrates increases the reactivity of each. In Table I, the effect of oleyl alcohol addition is more pronounced in stimulating palmitate esterification than esterification of the presumably more soluble linoleate. Palmitate esterification was greater when cholesterol was present together with oleyl alcohol but only a portion of this increase was due to stimulation of cholesterol esterification. Thus, the data of Table II show that oleyl alcohol increased the cholesterol esterified about 0.5 μ mole, from 335 to 2640 counts per minute, whereas total palmitate esterified was increased about 2.2 μ moles, from 345 to 9460 counts per minute, and oleyl alcohol esterification alone amounted to about 0.75 μ moles. The mere physical presence of cholesterol was capable of increasing oleyl alcohol reactivity (Table III) since the sterol is essentially unreactive in absence of taurocholate.

Pancreatic extracts prepared on different days showed considerable variation in degree of activity. Quantitative comparisons could only be made between reactions run at the same time, and data of each table are from single runs. They are representative of many experiments in which qualitative relationships were always the same. Since all extracts were prepared from the same lot of tissue in frozen storage, day-to-day variations may well be related to physical conditions.

The reactions described here proceeded quite as well anaerobically as aerobically and, in agreement with Pollard and Bieri(1), were not affected by treatment of the pancreatic extract with Dowex-1. In addition to the saturated and polyunsaturated acids used as representative fatty acid substrates in the experiments described, oleic acid also could serve. Thus, there is a close similarity to the vit. A esterifying system. Pollard and Bieri could find no requirement for taurocholate, and the effect of the bile salt on oleyl alcohol

esterification (Table III) is slight when compared with its action with the sterol.

The cholesterol-esterifying enzyme in pancreas was carried to a considerably higher degree of purity by Hernandez and Chaikoff (3). Although it was relatively non-specific for fatty acid substrates, they were able to elucidate several structural features of importance to the sterol specificity. If it is unlikely that the same pancreatic enzyme is catalyzing the esterification of several lipid-soluble compounds bearing a hydroxyl function, the experiments described here nevertheless establish an interrelationship.

Before the effects are regarded as solely an *in vitro* phenomenon, it is worthy of note that Channon and Collinson(6) observed that oleyl alcohol fed to rats increased the non-saponifiable liver lipids and a substantial portion of the increase was cholesterol. In terms of certain present-day concepts of cholesterol absorption(7), the presence of oleyl alcohol in the intestinal mucosa together with the pancreatic enzymes required for cholesterol esterification could facilitate esterification and, hence, absorption. In view of the similar reactivities of oleyl alcohol and vit. A, it is tempting to speculate that vit. A might similarly play a role in cholesterol esterification and absorption. Available information, however, makes it obvious that the vitamin is involved in sterol metabolism in a more complex relationship. Morton and associates (8) established that, under certain conditions, cholesterol feeding in rats may lower liver vit. A stores, but dietary vit. A deficiencies did not have direct effect on cholesterol levels. During these same studies, the first observations were made which adumbrated the finding that vit. A is involved in mevalonic acid utilization and other aspects of sterol metabolism(9,10).

Summary. Addition of oleyl alcohol to a pancreatic extract capable of cholesterol esterification resulted in esterification of the aliphatic alcohol and stimulation of cholesterol esterification. Correspondingly, the extent of the oleyl alcohol reaction was greater in presence of cholesterol. Some possible relationships of the findings to *in vivo* processes are discussed.

1. Pollard, C. J., Bieri, J. G., *Arch. Biochem. Biophys.*, 1960, v87, 9.
2. Swell, L., Treadwell, C. R., *J. Biol. Chem.*, 1955, v212, 141.
3. Hernandez, H. H., Chaikoff, I. L., *ibid.*, 1957, v228, 447.
4. Savary, P., Desnuelle, P., *Bull. Soc. Chim. Fr.*, 1954, 936.
5. Hirsch, J., Ahrens, E. H., *J. Biol. Chem.*, 1958, v233, 311.
6. Channon, H. J., Collinson, G. A., *Biochem. J.*, 1928, v22, 391.
7. Swell, L., Trout, E., Hopper, J., Field, H.,

- Treadwell, C. R., *J. Biol. Chem.*, 1958, v233, 49;
- Treadwell, C. R., Swell, L., Vahouny, G., Field, H., *J. Am. Oil Chemists' Soc.*, 1959, v36, 107.
8. Kantienga, N. L., Morton, R. A., *Biochem. J.*, 1955, v60, 28; Green, B., Lowe, J. S., Morton, R. A., *ibid.*, 1957, v67, 223, Green, B., Horner, A. A., Lowe, J. S., Morton, R. A., *ibid.*, 1957, v67, 235.
9. Gloor, U., Wiss, O., *Arch. Biochem. Biophys.*, 1959, v83, 216.
10. Van Dyke, R. A., Wolf, G., Johnson, B. C., *Biochem. Biophys. Research Commun.*, 1960, v3, 123.

Received January 6, 1961. P.S.E.B.M., 1961, v106.

Modification of Glucagon-Induced Hyperglycemia in Rats by 17-ethyl-19-nortestosterone. (26498)

ROBERT L. HAZELWOOD AND KEVIN D. O'BRIEN*

Dept. of Physiology, Boston University School of Medicine, Boston, Mass.

Considerable data are available indicating that 17-ethyl-19-nortestosterone (Nilevar) possesses potent myotrophic and anabolic properties as well as demonstrating a high anabolic to androgenic ratio(1,2,3). Recently, Nilevar was demonstrated to reduce the hyperglycemia associated with injection of glucagon (HGF) in human subjects(4). The specific mechanism by which Nilevar exerts its suppression of a glucagon-induced hyperglycemia is unknown. Blockade of glycogenolytic pathways, decreased gluconeogenesis, mediation through the pituitary or direct action on target endocrine tissues which affect any of the former, or a combination of the foregoing can be cited as the most likely possibilities.

Reported herein are studies carried out to determine more precisely the nature of Nilevar's reduction of glucagon-induced hyperglycemia. The ability of Nilevar to modify the glucagon tolerance test was compared with that of testosterone; additionally, castrated rats were compared with intact animals in their response to the hyperglycemic factor. Finally, hypophysectomized animals pretreated with Nilevar were subsequently in-

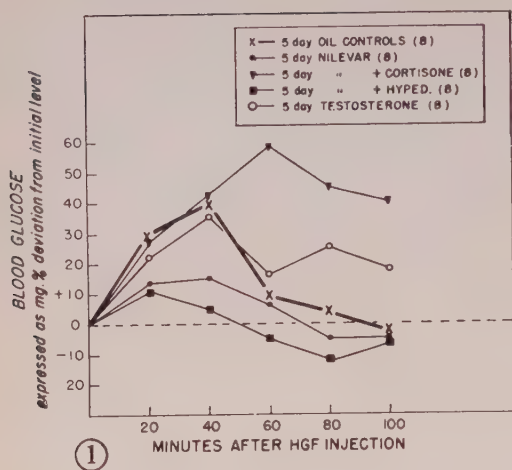
jected with glucagon to weigh the possible influence of hypophyseal secretions on the mode of action of Nilevar as an anti-hyperglycemic agent.

Methods. All rats employed in these studies were male descendants of the Sprague-Dawley strain, were fed Purina laboratory chow *ad libitum*, and were kept at a room temperature of 74-78°F. Rats, hypophysectomized at a body weight of 200-210 g, were purchased from the Charles River Breeding Laboratories and were used 20 days postoperatively. All injections were given daily at 9:00 a.m. for 5 consecutive days; the terminal injection was given 2 hours before taking the first blood sample. Both Nilevar and testosterone[†] were suspended in peanut oil such that 0.15 mg of either steroid was contained in 0.25 ml of vehicle. Control rats received peanut oil only in a volume equal to experimental groups. Cortisone acetate was suspended in saline such that 2.5 mg were contained in 0.25 ml.

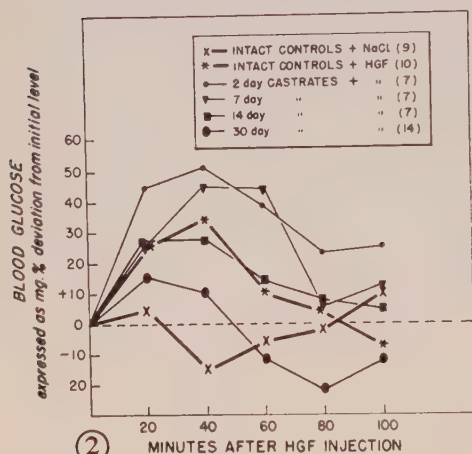
The glucagon tolerance test (GTT) was performed on rats fasted 4 hours (except in the study with hypophysectomized rats which were fed *ad libitum* to insure adequate liver

* Holder of a N.S.F. Medical Student Research Fellowship.

[†] Generously provided by Dr. V. A. Drill, G. D. Searle Co., Chicago, Ill.



①



②

FIG. 1. Glucagon tolerance tests in male rats inj. daily with 0.15 mg testosterone, with 0.15 mg Nilevar, or with 2.5 mg cortisone for 5 consecutive days. All groups were fasted 4 hr except the hypophysectomized rats which were fed *ad libitum*.

FIG. 2. Glucagon tolerance tests conducted in castrated male rats at varying post-operative periods.

glycogen) at the conclusion of which the animal was anesthetized with sodium pentobarbital (40 mg/kg body weight) and a midline ventral incision made. In some cases a very small piece of liver was taken for initial glycogen content. Serial blood samples (0.15 ml each) were obtained from the vena cava below the entrance of the renal vein. The control blood samples were taken immediately after injection of the crystalline glucagon solution† (0.1 mg/kg body weight) into the

vena cava and subsequent rinsing of the syringe with caval blood. Immediately after the final caval blood sample was drawn a terminal sample was taken from the abdominal aorta with the aid of a heparinized syringe to determine inorganic phosphorus and urea concentrations. Slices of liver and of the right testis were then quickly placed in tared test tubes containing 30% KOH for glycogen determination. The time from drawing the terminal aortic blood sample to the time the tissues were placed in KOH did not exceed 30 seconds.

Total reducing substances were determined on the serial caval blood samples using Nelson's colorimetric adaptation of the Somogyi method(5); serum inorganic phosphorus by the method of Sumner(6); urea by the diacetyl monoxime method of Rosenthal(7); and glycogen analyses were made by the anthrone method of Seifter *et al.*(8).

Results. Series I. Effect of Nilevar, testosterone or cortisone pre-administration on glucagon-induced hyperglycemia. The results are presented in Fig. 1 and Table I and clearly show that intact rats given a glucagon tolerance test (GTT) after a period of 5-daily Nilevar injections demonstrate a modified hyperglycemic response. Additionally, the anabolic steroid lowered blood urea levels significantly ($p < .01$). The presence of the pituitary gland was not essential for Nilevar to exert its anti-hyperglycemic effect as there were statistically significant ($p < .01$) differences between the blood glucose data of this group and those of the oil controls during the established peak of glucagon activity. Despite this anti-hyperglycemic effect, Nilevar was far less effective in increasing the body weight of the operated rats as compared with intact rats; also, there was no evidence of increased liver glycogen or decreased urea levels in the hypophysectomized animals as was seen in the intact rats receiving Nilevar.

Five daily injections of testosterone (at a dose identical with that of Nilevar) failed to modify the GTT in the intact rat. No significant differences were observed when blood glucose data of the oil control group were

† Generously donated by Dr. W. R. Kirtley, Eli Lilly Co., Indianapolis, Ind.

TABLE I. Effect of a Single Injection of Glucagon on Blood and Tissue Constituents of Rats Injected Daily for 5 Days with Nilevar, Testosterone and Cortisone.

Group	No. rats	Avg wt gain, g	Blood values in mg %		Glycogen as mg/g tissue	
			Phosphorus	Urea	Liver	Testis
Oil controls	8	+20	8.50 \pm 1.1*	44.7 \pm 3.8	13.6 \pm 4.2†	2.14 \pm .06
Nilevar (5 days)	8	+38	7.10 \pm .1	28.9 \pm 1.6	24.9 \pm 3.2	2.44 \pm .04
Testosterone (5 days)	8	+ 6	7.77 \pm .5	38.9 \pm 1.0	7.8 \pm 1.2	2.16 \pm .04
Nil. + cort. (")	8	- 8	6.67 \pm 1.2	51.5 \pm 1.2	60.2 \pm 5.4	2.22 \pm .03
Hypox + oil (")	5	0	4.93 \pm .3	65.6 \pm 2.3	24.4 \pm 5.1	2.53 \pm .12
" + Nil. (")	8	+ 3	6.51 \pm .3	71.1 \pm 3.1	5.2 \pm .4	2.36 \pm .06

* \pm stand. error.

† Pre-glucagon liver glycogen levels averaged 46.4 mg/g tissue for oil, Nilevar and testosterone groups; 80.2 mg/g for cortisone and Nilevar group; and 36.2 mg/g for hypophysectomized groups, both of which were non-fasted.

compared with those of the testosterone group. Far less increment in body weight occurred with testosterone than when either oil alone or Nilevar was injected, and blood urea, inorganic phosphorus and testicular glycogen appeared essentially unaltered (Table I).

When a daily dose of 2.5 mg of cortisone acetate was injected along with the standard dose of 0.15 mg Nilevar for 5 days, a super-normal response to the GTT lasting beyond the 100 minute observation period was observed. Also, in addition to this hypersensitivity to exogenous glucagon, blood urea and liver glycogen analyses indicate the failure of 0.15 mg Nilevar daily to suppress the powerful gluconeogenic action of the glucocorticoid.

Series II. Sensitivity of castrated male rats to exogenous glucagon. To test whether rats deprived of their normal endogenous supply of anabolic-androgenic secretions would demonstrate any altered response to the pancreatic hyperglycemic factor, groups of rats were castrated and subjected to a GTT at varying post-operative times. Hypersensitivity to exogenous glucagon is apparent in Fig. 2 where rats castrated 2 or 7

days previous to the GTT can be compared with intact controls ($p < .01$ at every point for the 2-day castrate group; $p < .01$ at the 40 and 60 min points for the 7-day castrate group). That this hypersensitivity to glucagon is transitory is apparent from the normal GTT response of the 2-week castrate group and by 30 days post-operatively there appears to be a definite resistance to HGF ($p < .05$, $< .01$, $< .01$ for the 40, 60 and 80 min blood samples respectively). Urea levels (Table II) increased significantly when compared with intact control values ($p < .01$) as early as 2 days post-operatively, returned to normal by the seventh post-operative day, only to rise once again by the thirtieth post-operative day ($p < .01$).

Discussion. As in man(4), Nilevar is an effective agent in reducing the hyperglycemic response evoked by exogenous glucagon in rats. Testosterone, however, appears to be without effect in this respect. It is unlikely that Nilevar-induced resistance to glucagon is mediated *via* hypophyseal activity, or even through the hypophyseal target areas, since the rats employed in these studies were hypophysectomized 20 days previously. Pro-

TABLE II. Effect of a Single Injection of Glucagon on Serum Inorganic Phosphorus, Urea, and Liver Glycogen in Castrated Male Rats.

Group	No. rats	Body wt, g	Blood values in mg %		Glycogen in mg/g tissue	
			Phosphorus	Urea	Liver	Testis
Intact controls + NaCl	9	183 \pm 9.1*	7.65 \pm .7	33.6 \pm 2.6	46.3 \pm 5.9†	2.35 \pm .06
" " + HGF	10	184 \pm 6.9	8.62 \pm 2.9	37.5 \pm .7	11.8 \pm 3.4	3.23 \pm 1.8
2 days castrate + HGF	7	161 \pm 7.4	7.82 \pm .4	52.8 \pm 1.9	16.7 \pm 3.1	—
7 " <i>idem</i>	7	180 \pm 2.4	8.64 \pm .7	39.0 \pm 1.5	9.0 \pm 2.5	—
14 " "	7	251 \pm 5.3	7.95 \pm .8	38.9 \pm 2.3	19.6 \pm 1.8	—
30 " "	14	313 \pm 4.6	7.41 \pm .2	48.2 \pm 1.3	17.7 \pm 3.3	—

* \pm stand. error.

† Pre-glucagon liver glycogen levels were in range of 38.5-49.1 mg/g tissue for all groups.

gressive postoperative atrophy of these tissues should render such an action partially or totally ineffective(9). Even though the GTT was modified in these operated animals, liver glycogen was grossly depleted indicating that blockade of glycogenolysis was not the mode of action of Nilevar in these preparations. That the protection against glucagon-induced hyperglycemia afforded by Nilevar may be mediated through gluconeogenic channels was evidenced by the sparing of protein concomitant with liver glycogen storage in the groups receiving Nilevar alone, as well as the exaggerated GTT curves, liver glycogen and urea levels in rats injected simultaneously with a gluconeogenic steroid such as cortisone. The hypersensitivity of the cortisone-injected rats to glucagon is in agreement with data reported on other species subjected to this double-hormone treatment(10).

Additional information as to the possible mechanism of action of Nilevar in curtailing the GTT was gained by testing the glucagon sensitivity of castrated male rats. The increased sensitivity of castrated rats to the hyperglycemic activity of HGF was transitory, the 2 and 7 days post-operative groups being hypersensitive, the 14-day castrates normal in their response, and 30-day post-operative group being refractive to exogenous glucagon. The explanation for the resistance which the one month castrate group demonstrated to glucagon is not readily available and is complicated by the fact that these animals paralleled the hypersensitive 2-day castrates in all other parameters studied (Table II). The early post-operative hypersensitivity to glucagon, in contrast to the ineffectiveness of injected testosterone to modify the GTT, would indicate that the mechanism whereby Nilevar exerts its protective influence is probably not mediated by a simple regulation of endogenous testosterone secretion.

The testicular glycogen data presented herein emphasize the stability of this carbohydrate moiety and are in accord with observations of other workers(11). Thus, a small but definite level of this polysaccharide exists in the rat testis and it must be concluded that it is of little importance in contributing to the hyperglycemia induced by exogenous glucagon.

Summary. Nilevar, but not testosterone, was effective in reducing the hyperglycemic response to exogenous glucagon in rats. Castrated rats were hypersensitive to the glucose elevating properties of glucagon for several days immediately after operation but by 30 days were resistant to the effects of glucagon. The refractory state induced by Nilevar probably was not mediated through the hypophysis, the hypophyseal target glands, or by blocking hepatic glycogenolytic pathways.

The excellent technical assistance of Barbara S. Hazelwood is gratefully acknowledged.

1. Barnes, L., Stafford, R., Guild, M., Thole, L., Olson, K., *Endocrinology*, 1954, v55, 77.
2. Spencer, H., Berger, E., Charles, M., Gottesman, E., Laszlo, D., *J. Clin. Endocrinol. and Metab.*, 1957, v17, 975.
3. Saunders, F., Drill, V., *Endocrinology*, 1956, v58, 567.
4. Weisenfeld, S., *PROC. SOC. EXP. BIOL. AND MED.*, 1958, v97, 764.
5. Nelson, N., *J. Biol. Chem.*, 1945, v160, 61.
6. Sumner, J. B., *Science*, 1944, v100, 413.
7. Rosenthal, J., *Anal. Chem.*, 1955, v27, 1980.
8. Seifter, S., Dayton, S., Novic, B., Muntwyler, E., *Arch. Biochem.*, 1950, v25, 191.
9. Turner, C. D., *General Endocrinology*, W. B. Saunders, Philadelphia, 1960.
10. Lazarus, S., Volk, B., Lew, H., *J. Clin. Endocrinol. and Metab.*, 1957, v17, 542.
11. Wolf, R. C., Leathem, J. H., *Endocrinology*, 1955, v57, 286.

Received January 9, 1961. P.S.E.B.M., 1961, v106.

Effect of 3, 4-Dichloroisoproterenol (DCI) on Body Temperature Changes Induced by Bacterial Endotoxin. (26499)

R. A. McLEAN AND L. JOE BERRY

Smith, Kline and French Labs., Philadelphia, and Bryn Mawr College, Bryn Mawr, Pa.

It is known that peripheral vasoconstriction occurs coincident with the onset of the pyrogenic reaction. Wells and Rall(1) suggested that the rise in body temperature resulted from a sudden and marked reduction in heat loss. They were later able to reduce and alter the febrile response in dogs with an adrenergic blocking drug(2). They induced fever in normal and in curarized dogs with intravenous doses of pyrogen obtained from *Pseudomonas aeruginosa*. N-ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr was their blocking agent. This compound is similar in mode of action to phenoxybenzamine. It blocks the alpha receptors according to the hypothesis of Ahlquist (3). A peripheral vasoconstriction, mediated by excitatory adrenergic responses in the skin, is regarded by Wells and Rall as the means by which heat loss is reduced and body temperature is raised. Kroneberg and Kurbjuweit(4) were able to inhibit bacterial pyrogen-induced fever in rabbits by pretreating the rabbits with reserpine. Iproniazid opposed the reserpine effect. This constitutes additional evidence that adrenergic mechanisms are involved but does not distinguish between excitatory or inhibitory factors.

The compound DCI or 3,4-dichloroisoproterenol is considered to be a specific blocker for inhibitory adrenergic responses(5,6). It was the only such drug which offered any protection against bacterial endotoxin described previously(7). Therefore, it was tested for ability to oppose the pyrogenic effects of *E. coli* endotoxin in unanesthetized rabbits.

Methods. Rectal temperature was measured with a thermistor in normal unanesthetized white rabbits restrained in an open stock. A "fever index" was calculated in the manner of Beeson(8) by taking as a base level the animals' temperature at time of injecting the pyrogen, and measuring with a

planimeter, the area enclosed between this line and the course of the elevated temperature. When temperature failed to return to the base level within the time limit a vertical line was drawn between the last temperature and the baseline. Fig. 1 illustrates this method. DCI and phenoxybenzamine solutions were made up in pyrogen-free injectable saline which was also used for the saline controls. These pretreatments were given to the rabbits subcutaneously 24 hours and 1 hour before endotoxin challenge. Difco crystalline lipopolysaccharide from *E. coli* (Code 0127:B8, Control 118023) was the endotoxin. It was also dissolved in pyrogen-free saline and given intravenously in the marginal vein of the ear. Similar observations were made in mice with endotoxin being given intraperitoneally.

Results. The results of these tests are summarized in Table I. Very little, if any, protection is apparent. The maximum rise in body temperature is the same in controls and DCI pretreated rabbits after 1.0 $\mu\text{g/kg}$ i.v. endotoxin. At a dose of 5 $\mu\text{g/kg}$ the control maximum was 0.4°F less than that of the pretreated rabbits but at 7 $\mu\text{g/kg}$, the opposite effect was seen. The DCI pretreated rabbits all had substantial fevers. In contrast, a group of rabbits pretreated in similar fashion with phenoxybenzamine experienced a very slight rise in body temperature.

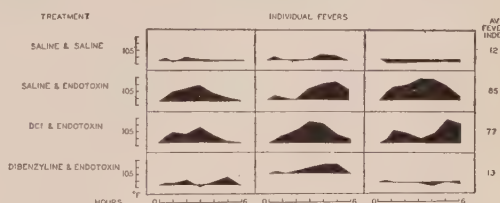


FIG. 1. Fevers in rabbits presented in the graphic manner of Beeson (1947). Degrees Fahrenheit are plotted on ordinates against time in hr on abscissae. Indices on right are obtained with a planimeter. 'Dibenzyliline' is the trade name for phenoxybenzamine.

TABLE I. Average Rectal Temperatures of Groups of Rabbits in Degrees Fahrenheit.

No. of rabbits	Pretreatment		Endotoxin dose, i.v. $\mu\text{g/kg}$	Test intervals after endotoxin in hr								Maximum effect
	Drug	Dose, mg/kg s.c.		Control	.5	1.0	2.0	3.0	4.0	5.0	6.0	
6	Saline blanks	—	(Saline)	103.7	103.4	103.3	103.4	103.4	103.6	103.6	103.5	— .4
2	" "	—	1	104.3	105.5	106.0	105.9	107.1	106.8	105.8	105.2	+2.8
3	DCI	2×10	1	103.8	104.7	105.2	105.2	106.6	106.6	105.6	105.7	+2.8
5	Saline blanks	—	5	103.3	103.9	104.3	104.5	105.7	105.7	105.0	104.1	+2.4
5	DCI	2×10	5	103.7	104.5	105.1	105.3	106.5	106.2	105.3	104.5	+2.8
3	Phenoxybenzamine	2×3	5	104.1	104.3	104.2	104.5	104.3	104.6	105.1	104.1	+1.0
3	Saline blanks	—	7	104.0	105.3	106.2	106.7	107.4	107.6	106.9	105.7	+3.6
3	DCI	2×10	7	103.5	104.2	104.6	105.4	106.2	106.1	105.2	104.0	+2.7

The effects of pretreatment with DCI and with phenoxybenzamine are also shown in Fig. 1. The first tier of areas are controls. Below this the fevers produced in 3 rabbits by an intravenous dose of 5 $\mu\text{g/kg}$ of endotoxin are portrayed. In order beneath these are similar responses in 3 rabbits pretreated with 2 doses of DCI 10 mg/kg, given 24 hr and 1 hr before the endotoxin. The lower tier clearly shows the antipyretic effect of phenoxybenzamine. This was to be expected from the report by Wells and Rall. Although the fever index is somewhat lower in the DCI pretreated animals than in those that received saline before endotoxin this difference is negligible when compared with the phenoxybenzamine effect. Similar comparisons were made between saline and DCI pretreated rabbits at doses of 1 and 7 $\mu\text{g/kg}$ i.v. endotoxin. The fever indices in the first case were 62 (saline) vs. 63 (DCI) and 102 vs. 84 in the second. Although fever indices in the DCI pretreated rabbits were lower at both the 5 $\mu\text{g/kg}$ and 7 $\mu\text{g/kg}$ doses of endotoxin and a slight protective effect may be suggested it is not considered adequate to explain previous findings(7). Here phenoxybenzamine failed to offer protection against the lethal effect of bacterial endotoxin while DCI was clearly beneficial. In these rabbit tests phenoxybenzamine was markedly antipyretic and DCI was not.

Berry, Smythe and Young(9) had noted hypopyrexia rather than fever when large doses of endotoxin were given to mice. It seemed possible that their effects might be the result of heat loss through vasodilation.

Such an effect would be opposite that proposed by Wells and Rall. If so, it might be susceptible to inhibition by DCI. To investigate this possibility 2 series of body temperature measurements were made in mice treated and untreated before endotoxin administration. These are presented in Fig. 2. Although average temperatures for the saline control

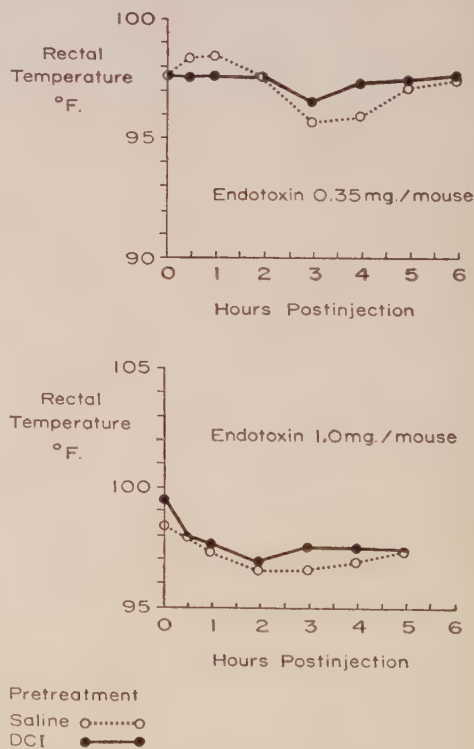


FIG. 2. Rectal temperatures of mice after intraper. inj. of 0.35 or 1.0 mg *E. coli* endotoxin. Each point is the avg of at least 10 determinations. DCI dose is 2×10 mg/kg s.c. (24 hr and 1 hr) before the endotoxin.

groups were slightly lower than comparable figures for DCI pretreated animals statistical treatment indicated that the apparent differences were not at all significant.

It would appear from this series of experiments that the unique type of adrenergic blockade produced by DCI(5,6) is not effective against either fever or heat loss. It is concluded that some mechanism other than body temperature is concerned in the protective action of DCI which has been observed in mice challenged with *E. coli* endotoxin(7).

These data also suggest that the pyrogenicity of an endotoxin is not directly correlated with its toxicity. This is an important observation since many investigators use as an assay for endotoxin its ability to produce hyperthermia in experimental test animals. If the assay employed fails to measure the originally significant property of an endotoxin, *i.e.*, its toxicity, then the value of the assay needs thoughtful evaluation.

Summary. 1) Pretreatment of rabbits

with DCI failed to prevent the pyrogenic reaction to injected bacterial endotoxin. 2) The hypopyrexia produced in mice by endotoxin likewise was unaffected. 3) It is concluded that the type of adrenergic inhibition produced by this substance is not germane to the problem of body temperature changes induced by bacterial endotoxins.

1. Wells, J. A., Rall, D. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, v68, 421.
2. ———, *ibid.*, 1949, v70, 169.
3. Ahlquist, R. P., *Am. J. Physiol.*, 1948, v153, 586.
4. Kroneberg, G., Kurbjuweit, H. G., *Arzneimittel Forsch.*, 1959, v9, 556.
5. Powell, C. E., Slater, I. H., *J. Pharm. Exp. Therap.*, 1958, v122, 480.
6. Moran, N. C., Perkins, M. E., *ibid.*, 1958, v124, 223.
7. McLean, R. A., Berry, L. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v105, 91.
8. Beeson, P. B., *J. Exp. Med.*, 1947, v86, 29.
9. Berry, L. J., Smythe, D. S., Young, L. G., *ibid.*, 1959, v110, 389.

Received January 11, 1961. P.S.E.B.M., 1961, v106.

Partial Replacement of Serum and Embryo Extract Requirements for Growth of Avian Cell Cultures.* (26500)

ROBERT E. NEUMAN AND ALFRED A. TYTELL

Virus and Tissue Culture Research Division, Merck Institute for Therapeutic Research, West Point, Pa.

Embryo extract has been employed extensively in tissue culture(1) since the discovery of its stimulatory effects by Carrel(2). Modern developments have eliminated this material in a majority of instances from the requirements of mammalian cells(3); nevertheless, avian cultures, upon which the initial observations were made, have continued to require embryo extract or components of embryo extract for satisfactory growth(4,5,6). Serum, traditionally necessary for mammalian cells, also has been essential to avian cultures in addition to the embryo extract(4,5,6).

The present report concerns the growth re-

sponse of avian cultures to constituents of a serumless medium devised for cultivation of mammalian cells(7).

Methods and materials have been described previously(6). In brief, cell suspensions of 14-16 day white Leghorn chick embryo lung or heart were prepared by stirring the minced tissue 2-4 hours with 0.2% Difco 1:250 trypsin in Earle's balanced salt solution at 37°C. The cell suspension filtered through gauze and collected by centrifugation was resuspended in a complete medium containing serum and embryo extract(6). One ml amounts of the suspension containing 50,000 to 100,000 cells per ml were pipetted into 15 × 125 mm roller tubes placed in a slanted position in racks. The cultures were established

* This work was completed under contract with Cancer Chemotherapy National Service Center, Nat. Cancer Inst., N.I.H., U.S.P.H.S.

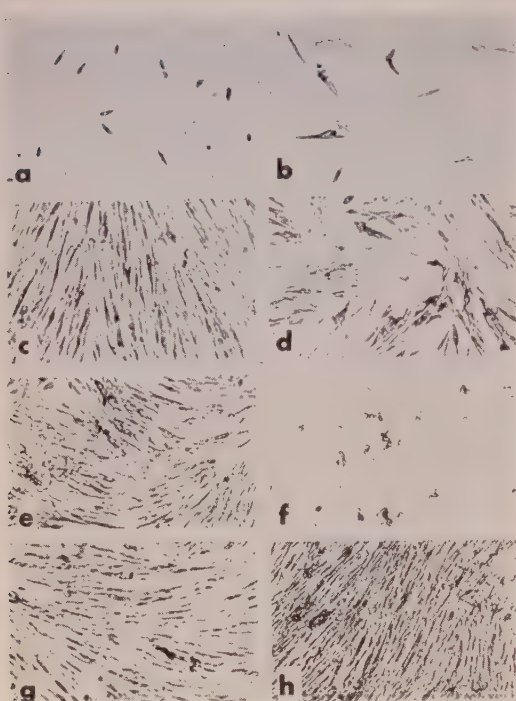


FIG. 1. Response of chick embryo lung cells to different media. Growth period, 4 days. Cells fixed with Bouin's and stained with Giemsa. $\times 30$. a. Plated inoculum. b. Basal medium A. c. Basal medium A with 5% dialyzed bovine serum and 5% dialyzed embryo extract. d. Basal medium A plus special additives. e. Basal medium A plus special additives and 5% dialyzed bovine serum. f. Basal medium A plus special additives plus 5% dialyzed serum, no lactalysate residue. g. Basal medium A plus special additives, 5% dialyzed serum and 5% dialyzed embryo extract, no lactalysate residue. h. Basal medium A plus special additives, 5% dialyzed serum and 5% dialyzed embryo extract, lactalysate residue included.

(plated) overnight by incubation at 37°C in an atmosphere of 8% carbon dioxide—92% air. The medium was removed and the tubes rinsed with Earle's solution. The cells then were overlaid with one ml of basal medium containing graduated quantities of test materials. After further incubation for 3-5 days growth was measured by the protein determination method of Oyama and Eagle(8). The basal test medium (Medium A) for these studies consisted of all the constituents of the serumless medium for mammalian cultures(7) with the exception of the special additives which were incorporated as indicated for individual experiments. The preparation of dialyzed bovine serum and dialyzed em-

bryo extract used has been described(6).

The relatively non-dialyzable portion of lactalysate[†] (lactalysate residue) was prepared by dialysis overnight against running tap water followed by dialysis for 24 hours against several changes of an excess of demineralized water. The non-dialyzable residue then was freeze-dried. Hydrolysates of lactalysate residue were prepared by autoclaving 100 mg in 5 ml 6 N HCl in sealed tubes for 16 hours at 15 lb. pressure. HCl was removed by flash evaporation. The hydrolysate was taken up in ethanol and again flash evaporated. An aqueous solution of the hydrolysate was then decolorized with Darco, grade S-51.[‡]

Results. The appearance of typical chick embryo lung cultures in various media is shown in Fig. 1. The plated cells of the inoculum (Fig. 1a) increased in size but did not multiply in Medium A (Fig. 1b). Supplementation with dialyzed embryo extract and dialyzed serum promoted rapid growth (Fig. 1c). Addition of pyruvate, insulin, salmine, lactalysate (or derived preparations), folic acid and methyl oleate, which were special constituents of serumless medium, enabled the cells to grow into sheets (Fig. 1d). Addition of dialyzed serum to the medium containing the special additives produced rapid growth (Fig. 1e) comparable with media containing embryo extract and serum. In the presence of serum, omission of lactalysate or derivatives resulted in a marked toxicity (Fig. 1f). Embryo extract prevented this toxicity (Fig. 1g) as did the lactalysate preparations and good growth proceeded in medium containing embryo extract, serum, and special additives together with the non-dialyzable residue of lactalysate (Fig. 1h). Inclusion of whole lactalysate in the most complex medium often resulted in a characteristic clumping of cells and less growth. In all further studies lactalysate residue was used rather than whole lactalysate.

Investigation of the effect of special constituents added singly or in combination to

[†] A pancreatic digest of lactalbumin, Edamin DR, Sheffield Chemical Co., Norwich, N. Y.

[‡] A. H. Thomas Co., Philadelphia, Pa.

TABLE I. Effect of Insulin, Salmine, Pyruvate, and Lactalysate Residue on Growth of Chick Embryo Heart and Lung Cell Cultures.

Insulin			Salmine			Pyruvate			Lactalysate residue		
μg cell prot./tube			μg cell prot./tube			μg cell prot./tube			μg cell prot./tube		
0	5%		0	5%		0	5%		0	5%	
$\mu\text{g}/\text{ml}$	serum	serum	$\mu\text{g}/\text{ml}$	serum	serum	mM	serum	serum	$\mu\text{g}/\text{ml}$	serum	serum
Chick embryo heart											
0	4	138	0	30	48	0	30	102	0	22	18
.05	10	121	.1	30	74	.01	36	116	25	54	56
.1	10	121	.5	38	46	.05	36	138	50	44	98
.5	20	138	1	38	84	.1	32	166	100	98	144
1	36	150	3	44	166	.2	38	168	200	52	184
2	34	178	5	38	172	.5	42	182	500	42	190
5	40	190	8	38	166	1	46	184	1000	46	186
10	46	178	10	44	154	2	48	164			
Chick embryo lung											
0	36	82	0	96	36	0	100	132	0	12	12
.05	38	80	.1	96	40	.01	112	128	25	28	42
.1	38	80	.5	112	40	.05	118	126	50	48	72
.5	46	88	1	118	78	.1	108	128	100	60	92
1	52	94	3	126	156	.2	118	130	200	60	104
2	54	102	5	118	170	.5	118	152	500	68	128
5	58	116	8	102	182	1	112	158	1000	66	118
10	64	88	10	104	158	2	118	162			

Inoculum was 80,000 cells/ml. Growth was 4 days at 37°C in basal medium A together with insulin, salmine, pyruvate, lactalysate residue, folic acid and methyl oleate with and without 5% dialyzed serum.

medium A with and without serum demonstrated the stimulatory effects of lactalysate and derivatives, pyruvate, insulin, and salmine. Methyl oleate and folic acid were not found to be consistently stimulatory. Mucic acid and galacturonic acid at no time appeared beneficial and were omitted from the media. In Table I is shown the growth response of chick embryo lung and heart cultures to graduated quantities of insulin, salmine, pyruvate and lactalysate residue. The basal medium was medium A, containing, unless otherwise specified, 5 μg insulin, 5 μg salmine sulfate, 250 μg lactalysate residue, 1 mM pyruvate, 0.1 μg folic acid, and 5 μg methyl oleate per ml with and without 5% dialyzed bovine serum. It will be noted that omission of any one of the additives evaluated resulted in decreased growth. Optimal levels were approximately the quantities included in the complete medium (as listed above) and which were determined by preliminary experiment. The heart and lung cultures responded in a similar fashion. Growth was greatest in media containing serum. Variation of the serum concentration over a range of 3 to 10% did not affect results appreciably.

The role of lactalysate or derivatives was

especially important in that these overcame the toxicity noted previously, which was characteristic of all samples of bovine serum from animals of relative maturity or chicken serum tested whether dialyzed or undialyzed. This toxicity of serum was manifested in the simpler medium A as well as in presence of the special constituents. In either case lactalysate, its derivatives, or embryo extract overcame the toxicity. On occasion, lactalysate was not wholly satisfactory in media containing samples of serum which were exceptionally toxic. Examination of the lactalysate revealed that stimulatory and protective factor(s) reside chiefly in the non-dialyzable portion comprising about 8% of the total. This "lactalysate residue" at 250 $\mu\text{g}/\text{ml}$ substituted completely for 1-5 mg of lactalysate to stimulate growth and consistently overcame toxicity of *all* sera tested. Although the non-dialyzable property of the active portion of lactalysate suggested a proteose or peptone, this concept was dispelled by the equivalent activity of lactalysate residue subjected to acid hydrolysis (Table II). Identification of the active factor(s) is being investigated.

Bacto-peptone displayed activity but was

TABLE II. Relative Activity of Lactalsate, Lactalsate Residue and Its Acid Hydrolysate for Growth of Chick Embryo Lung Cultures.

Lactalsate		Lactalsate residue		Lactalsate residue (acid hydrolysate*)	
mg/ml	μg cell protein/tube	mg/ml	μg cell protein/tube	mg/ml	μg cell protein/tube
0	12	0	12	0	12
.1	12	.025	106	.025	102
.5	30	.05	138	.05	128
1.0	60	.1	172	.1	158
2.0	100	.2	186	.2	166
3.0	122	.5	194		
5.0	142	1.0	186		

* Autoclaved 16 hr in 6 N HCl. Inoculum was 80,000 cells/ml. Growth was 4 days at 37°C in basal medium A with special additives and 5% dialyzed bovine serum.

much less potent than lactalsate. Bactotryptone and Difco proteose-peptone were not significantly active.

Bovine fetal serum containing essentially a gamma protein which was 39% α -globulin,[§] presumably largely fetuin, proved to be an exception among all sera tested in that it was non-toxic. Table III gives a comparison of growth of chick embryo lung cultures in media containing dialyzed fetal calf serum and dialyzed bovine serum. The contrast in toxic properties of the 2 sera may be noted. Addition of lactalsate or embryo extract was unnecessary for maximal growth with fetal serum. However, growth was inferior to that in media containing either of these factors together with the customary bovine serum. Fetuin stimulated growth in absence of serum as did the lactalsate residue or embryo extract, although it did not overcome toxicity of bovine serum. The effectiveness of fetal serum apparently is not attributable to its high content of fetuin.

Relative growth rates of chick embryo lung cultures on different media are shown in Fig. 2. Inclusion of the special additives with medium A accelerated growth. Further addition of 5% dialyzed serum supported growth rates comparable to those in media containing 5% dialyzed serum and 5% dialyzed embryo extract.

Freshly prepared chick embryo lung cells were found to plate out and grow in media containing the special additives and 5%

dialyzed serum without previous establishment in media containing embryo extract. These cells were grown through several subcultures. Growth rates declined, however, and it became apparent that surviving cells would represent a selected or adapted population, and the cultures were abandoned.

Discussion. Of particular interest in growth of the avian cultures were the beneficial effects of the special additives even in presence of serum. Salmine, which enables cells to adhere to surfaces of culture vessels (9), possibly complemented the action of suboptimal concentrations of naturally occurring "sticking" or "spreading" factors. Insulin may also be required in relatively high

TABLE III. Comparison of Growth of Chick Embryo Lung Cultures in Media Containing Dialyzed Fetal Calf Serum and Dialyzed Bovine Serum.

Dialyzed bovine serum (%)	Addition to basal medium: *			
	None	250 μg lactalsate residue/ml	2 mg fetuin/ml	5% Embryo extract
		(μg cell protein/tube)		
0	16	114	74	64
2	38	158	92	172
5	36	202	56	178
10	46	224	44	206
Dialyzed bovine fetal serum (%)				
0	16	108	82	58
2	130	140	114	136
5	148	138	132	118
10	146	166	146	128

Inoculum was 80,000 cells/ml. Growth was for 4 days at 37°C.

* Basal medium A containing insulin, pyruvate, salmine, oleate and folic acid.

[§] Obtained from Colorado Serum Co., Denver, Paper electrophoretic analysis by Dr. A. I. Schepartz, Merck, Sharp and Dohme.

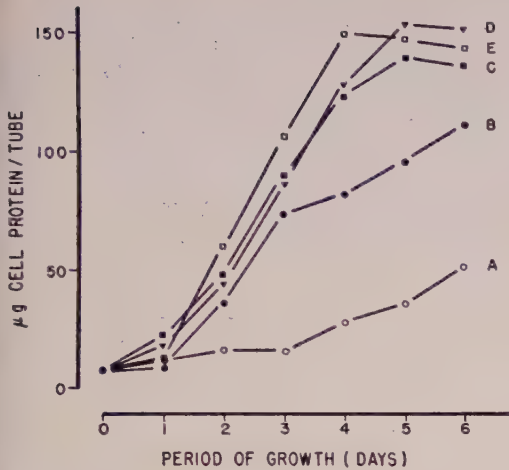


FIG. 2. Growth rates of chick embryo lung culture in different media. A. Basal medium A. B. Basal medium A plus special additives. C. Basal medium A plus 5% dialyzed bovine serum and 5% dialyzed embryo extract. D. Basal medium A plus special additives and 5% dialyzed bovine serum. E. Basal medium A plus special additives, 5% dialyzed bovine serum, 5% dialyzed embryo extract, no lactalsate residue.

concentrations for maximal growth rates under *in vitro* conditions. Pyruvate promotes growth of isolated Walker carcinosarcoma 256 cells(10) and cells in serumless media (7). Oleate and folic acid did not significantly stimulate growth presumably due to their presence in the serum or to metabolic capacities of the cells. Lactalsate apparently not only provided additional growth factor(s) as indicated by stimulation either in presence or absence of serum, but it also functioned specifically to overcome severe toxic effects of serum. Embryo extract, too, was highly effective in preventing serum toxicity which may account in part for the requirement for this material. Toxic fractions have been prepared from serum by Cohn fractionation procedures(11). Fetal calf serum was observed to be non-toxic although its growth-supporting potential was inferior to other bovine serum.

Conceivably, unusual specimens of serum from more mature animals but containing maximal concentrations of sticking factors and other stimulatory substances and minimal toxic substances would support satisfactory growth of avian cells in absence of em-

bryo extract, lactalsate or derivatives. Harris and Kutsky grew cultures of chick myoblasts which required both serum and nucleoprotein prepared from embryo extract(5). Subsequently, Harris grew myoblasts in a specimen of serum to which the addition of embryo extract or nucleoprotein was unnecessary(12). He further demonstrated a requirement for the dialyzable portion of the serum or for Bacto-peptone. In the present studies the sera tested with the exception of fetal calf serum, whether of chicken or bovine origin, whole or dialyzed, supported satisfactory growth of the chick lung or heart cells only in presence of embryo extract, lactalsate or its derivatives. Serum dialysates were not found to be active. Limited growth could be obtained without embryo extract or lactalsate factor(s) by employment of large inocula (300,000/ml), perhaps due to conditioning of the medium. Proteose-peptones were found to be highly stimulatory as substitutes for embryo extract for growth of explants in plasma clots(13). An anti-toxic action was not ascribed to such materials. Although in the present studies the activity of lactalsate was concentrated in the non-dialyzable portion, the capacity of the lactalsate residue to stimulate growth and to overcome toxicity of serum was not destroyed by acid hydrolysis. Evidently the active factor(s) was not peptone in character.

Summary. Salmine, pyruvate, insulin and lactalsate or its derivatives have been shown to replace partially requirements of avian cell cultures for serum and embryo extract. In addition to these factors, however, serum was required for growth comparable to that attained in the basal medium containing both serum and embryo extract. A function of lactalsate and embryo extract was inhibition of a toxic effect of serum. The stimulatory and "anti-toxic" effects of lactalsate were confined largely to the non-dialyzable portion. Nevertheless, the activity of acid hydrolysates of the lactalsate residue demonstrated that these properties were not dependent on a proteose or peptone.

We wish to acknowledge the excellent technical assistance of Miss Joan L. Molowski.

1. *Methods of Tissue Culture*, Parker, R. C., Paul B. Hoeber, Inc., N. Y., 2nd edition, 1950.
2. Carrel, A., *J. Exp. Med.*, 1913, v17, 14.
3. *Handbook of Cell and Organ Culture*, Merchant, D. J., Kahn, R. H., Murphy, W. H., Jr., Burgess Publishing Co., Minneapolis, Minn., 1960.
4. Kutsy, R. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1953, v83, 390.
5. Harris, M., Kutsy, R. J., *Cancer Res.*, 1958, v18, 585.
6. Neuman, R. E., Tytell, A. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v103, 71.

7. ———, *ibid.*, 1960, v104, 252.
8. Oyama, V. I., Eagle, H., *ibid.*, 1956, v91, 305.
9. Lieberman, I., Ove, P., *J. Biol. Chem.*, 1958, v233, 637.
10. Neuman, R. E., McCoy, T. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1958, v98, 303.
11. Chang, R. S., Pennell, R. B., Keller, W., Wheaton, L., Liepens, H., *ibid.*, 1959, v102, 213.
12. Harris, M., *ibid.*, 1959, v102, 468.
13. Carrel, A., Baker, L. E., *J. Exp. Med.*, 1926, v44, 503.

Received February 9, 1961. P.S.E.B.M., 1961, v106.

Racial Variations in Sweat Gland Distribution. (26501)

AIKOH KAWAHATA* AND THOMAS ADAMS (Introduced by L. D. Carlson)
Dept. of Physiology and Biophysics, University of Washington School of Medicine, Seattle

Ogata(1) reported the existence of both active and inactive sweat glands in man. Although no difference between these 2 types of glands is seen histologically, only the active glands appear to be stimulated to secretion by either pharmacological agents or thermal stress. Because the number of sites of secretions was used as an index of the number of sweat glands, the data reported here are assumed to be applicable to only the active sweat glands, by Ogata's distinction.

In Japanese subjects Kawahata(2) showed that the number of sweat glands is smaller at birth than at 2 years of age, and suggested that initially inactive glands may be recruited into use during the first years of life. In support of this suggested environmental influence on eventual number of sweat glands, he subsequently demonstrated that Japanese born and living in tropical areas have a greater number, and consequently a greater density of sweat glands than members of the same racial group inhabiting temperate zones. Seemingly further weight is added to these suggestions by Kawahata's finding that Caucasian (Russian) subjects living in northern Manchuria have fewer active sweat glands, despite larger average body size, than do Japanese from temperate climates. In making the

latter comparison, however, Kawahata noted that possible racial differences might have influenced these data. Kawahata and Sakamoto(3) have shown that Ainos in Hokkaido, Japan, have fewer sweat glands than do Japanese inhabiting the same island. Both factors modifying sweat gland distribution, racial and environmental, merit additional study.

Methods. The subjects sampled consisted of male Negroes and male and female Caucasians and Eskimos. The Negro subjects were born and lived in the midwestern United States. The origins of the Caucasian group were varied as shown in Table II. The Eskimo group was sampled in their native village (Anaktuvak Pass) in the Brooks mountain range in interior Alaska. The ages of all subjects are reported with the observed sweat gland data in Tables I to III.

Sweat gland measurements were made in heated chambers maintained at 41°-42°C after the subject had been in the room for 30-50 minutes and was sweating maximally. Sweat glands were counted by Jurgensen's method as modified by Kawahata(2). This method consists of counting under magnification (10×) the sites of sweat secretion on the skin surface covered with tinted cedar oil. The secretion sites corresponding to the openings of the underlying glands are counted within a 0.09 cm² area stamped onto the skin

* Current address: School of Med., Mie Prefecture University, Torii-cho, Tsu, Japan.

TABLE I. Male Caucasian and Male Negro.

Subj. No.	Age (yr)	Total sweat glands ($\times 10^3$)	No. of sweat glands (per cm^2)
A. Male Negro (mean S.A. = 1.86 M^2)			
1	25	2024	115
2	20	2046	109
3	21	2058	114
4	22	2138	109
5	23	2199	124
6	31	2203	125
7	20	2256	132
8	19	2313	136
9	36	2371	100
Mean \pm S.E.		2179 ± 115	117 ± 11
B. Male Caucasian (mean S.A. = 1.88 M^2)			
1	25	1800	92
2	26	2304	125
3	21	2364	119
4	21	2369	130
5	23	2405	126
6	16	2523	139
7	21	2579	147
8	18	2640	126
9	22	2815	179
10	24	2894	128
Mean \pm S.E.		2469 ± 290	131 ± 21

before application of the oil. Estimation of active glands depends on averaging sample points(3-5) at 20 different skin regions over the body. Eight regions were used for the Eskimo group (forehead, cheek, upper and lower arm and leg, and dorsum of hand and foot). From these data both total number and distribution of active sweat glands may be estimated. The surface area for each meas-

TABLE II. Female Caucasian.
(Mean S.A. = 1.63 M^2)

Subj. No.	Age (yr)	Total sweat glands ($\times 10^3$)	No. of sweat glands (per cm^2)	Geographical origin
1	19	2478	170	Denmark
2	22	2501	171	Germany
3	21	2572	147	U.S.A.
4	22	2663	182	Germany
5	20	2849	154	U.S.A.
6	18	2984	178	"
7	19	3010	182	"
8	19	3069	185	"
9	20	3111	200	Germany
10	18	3155	184	U.S.A.
11	20	3254	183	Turkey
12	25	3279	221	"
13	20	3533	204	U.S.A.
14	17	3551	219	Turkey
15	24	3611	240	"
16	21	3679	211	Holland
17	29	3827	236	Hungary
Mean \pm S.E.		3123 ± 410	192 ± 25	

TABLE III. Eskimo.

Subj. No.	Age (yr)	Total sweat glands ($\times 10^3$)	No. of sweat glands (per cm^2)
A. Male Eskimo (mean S.A. = 1.77 M^2)			
1	27	1727	102
2	44	2077	118
B. Female Eskimo (mean S.A. = 1.58 M^2)			
1	18	2452	157
2	23	1771	105
3	20	2592	165
4	14	2725	192
5	16	3068	206
6	35	2476	155
7	29	1934	124
8	36	2071	141
Mean		2386	156

ured body region was derived from a linear formula suggested by DuBois and modified by Kawahata(4). Whole body surface areas for the Eskimo group were estimated from height and weight, according to the method of DuBois(5). The accuracy and reproducibility of measurements of active sweat glands during maximal sweating have been confirmed by Randall(6).

Results. Tables I to III contain the data for all subjects. The density and total number of sweat glands for Caucasian men exceeds those for the Negro subjects ($P < 0.02$),[†] even though these 2 groups have similar average whole body surface areas (1.88 and 1.86 m^2) (Table I). Further, comparison of these data with those in Table II shows that Caucasian women exceed both Caucasian and Negro men in these measures ($P < 0.01$). Even though average whole body surface areas (1.63 m^2) for the Caucasian female subjects are smaller ($P < 0.01$) than those of the males of the same race (1.88 m^2), the total number of glands appears to be greater ($P < 0.01$).

Both of these measurements are smaller for the Eskimo than for the Caucasian women ($P < 0.05$) whereas no apparent differences ($P > 0.05$) exist either within the Eskimo group or between these two races for male subjects, although the sampling of Eskimo men was too small to support conclusive statements (Table III).

Discussion. According to Randall(6)

[†] Fisher T test.

higher environmental temperatures are required to produce observable sweat secretions from normal glands than from those affected by mecholyl iontophoresis. He suggests on this basis that the "inactive glands" described by Ogata(1) were simply not responding to the thermal stimulus. Ogata reported earlier, however, that the same total number and distribution of sweat glands were measured with either extreme heat or locally injected pilocarpine. Using injected acetylcholine instead of pilocarpine, Kawahata(7) obtained similar results.

Other evidence also argues against the assumption that less active sweat glands may be mistaken for inactive glands in these measurements(8). These data show that sweat production follows predictably with increasing degrees of locally applied thermal stress, although total number or position of active glands does not change.

Since a tropical environment influences early activation of sweat glands, it might appear surprising that the Caucasian males showed a greater number of activated sweat glands than did the male Negroes, members of a racial group with essentially a tropical origin. However, the Negro subjects in this study were born and reared in temperate climates and these physiological measurements might more predictably reflect environmental than genetic factors. This might also be pertinent to an interpretation of Thomson's report(9) of no differences in sweat gland density between native Negroes and immigrated Caucasians in tropical Africa. These data, however, may not have been gathered under conditions of maximal sweating, as indicated by Kuno(10). Ages of the Caucasians at time of immigration must be considered. In view of the similarities of environmental influences on Negro and Caucasian groups in this study, the reported differences in sweat gland density most reasonably appear to be of a racial origin.

The sex difference in sweat gland distribution in the Caucasian group may appear paradoxical considering the observations of Tanaka(11) and others(12-15) that the male Caucasian response to thermal sweating is

greater than that of the female Caucasian. However, this difference may be one of response threshold to stimulation and activity level of the activated sweat gland and not one of distribution. Additional data on other racial groups must be obtained before these observations can be attributed to a sex difference, rather than to environmental or other factors.

It is interesting that the measurements indicate that Eskimo women had fewer active sweat glands than female Caucasians have; however, both environmental and racial effects may be operating. The efficient micro-environment of the Eskimo even in the extreme thermal stress of their natural climate must be considered before these differences are attributed to environmental factors.

Summary. Total number and distribution of active sweat glands were determined for American Negro, Caucasian and Eskimo subjects under conditions of maximal thermal sweating. Both sexes were included in Caucasian and Eskimo groups, whereas only adult, male Negro subjects were investigated. A greater density and total number of active sweat glands were found in Caucasian males than in Negro males; Caucasian women appear to exceed any other group in these measurements, regardless of race or sex. The measurements in Eskimo women did not follow this pattern and they appear to be like the others measured.

The authors wish to express appreciation to Dr. Irvin M. Korr, Kirksville College of Osteopathy and Surgery for generous help and critical advice, to Dr. Walter C. Randall of Loyola University for affording facilities, and to Dr. Russell Seckendorf for cooperation.

1. Kuno, Y., *Human Perspiration*, Charles C. Thomas, Springfield, Ill., 1956, p57.
2. Kawahata, A., *J. Mie Med. Coll.*, 1950, v1, 25.
3. Kawahata, A., Sakamoto, H., *Jap. J. Physiol.*, 1951, v2, 166.
4. Kawahata, A., *J. Physiol. Soc. Japan*, 1940, v5, 245.
5. DuBois, D., DuBois, E. F., *Arch. Int. Med.*, 1915, v15, 868.
6. Randall, W. C., *J. Clin. Invest.*, 1946, v25, 761.
7. Kawahata, A., unpublished data.

- 8.——, *J. Mie Med. Coll.*, 1950, v1, 123.
9. Thomson, M. L., *J. Physiol.*, 1954, v123, 225.
10. Kuno, Y., *Human Perspiration*, Charles C Thomas, Springfield, Ill., 1956, p63.
11. Tanaka, M., *Mie Med. J.*, 1957, v7, 109.
12. Gibson, T. E., Shelley, W. B., *J. Inv. Dermat.*, 1948, v11, 137.
13. Janowitz, H. D., Grossman, M. I., *ibid.*, 1950, v14, 453.
14. Shelley, W. B., Horovath, P. N., Pillsbury, D. M., *Medicine*, 1950, v29, 195.
15. Herman, F. J., Prose, P. H., Sulzberger, M. B., *J. Inv. Dermat.*, 1952, v18, 71.

Received February 17, 1961. P.S.E.B.M., 1961, v106.

Embolie Trophoblast in Peripheral Circulation During Pregnancy.* (26502)

BENJAMIN L. TOY AND LUKE G. TEDESCHI (Introduced by C. G. Tedeschi)

*Department of Laboratories and Research, Framingham Union Hospital, Framingham, Mass., and
Departments of Pathology and Surgery, Massachusetts Memorial Hospitals, Boston*

Pulmonary embolism by trophoblastic fragments during pregnancy was first demonstrated by Schmorl(1), and is now considered a commonplace finding in normal gestation. Park(2), as well as Bardawil and Toy (3) have implied that one may anticipate uncomplicated trophoblastic embolism to the lungs in roughly half of all seemingly physiological pregnancies. The fate of ectopic trophoblasts has aroused particular interest as a possible factor in the genesis of chorio-carcinoma. Experiments such as those by Park(2) have suggested a destiny no more threatening than ultimate degeneration and disappearance from the host.

Until recently, free trophoblastic embolism has been observed only in the pulmonary capillaries. Douglas *et al.*(4) recently reported isolation of syncytial masses at time of Caesarean section from the veins of the broad ligament in 8 of 13 cases, the ovarian veins of one patient, and the inferior vena cava in 3 samples from an unstated number of cases out of a total of 33 explored. Attempts to isolate trophoblasts from the antecubital vein failed to reveal positive results. The desirability of a statistical perspective on the problem of trophoblastic embolism and its analysis has induced the authors to submit their results of a similar survey.

Materials and methods. Blood samples

were drawn from a total of 155 patients in various stages of pregnancy, 10 of whom were in active labor at time of sampling. Specimens were obtained in all cases from the antecubital vein and in 22 of these patients blood was also secured from the placental site during Caesarean section. Ten smears and a serially sectioned cell block were examined in each of the first 130 cases; 5 smears were evaluated in each of the remaining 25 cases. The latter included 5 samples from the placental site.

Early attempts to apply the method of Sandberg and Moore(5) yielded unsatisfactory results. The majority of samples have been processed according to the technic of Malmgren and his group(6) as later modified by Long and his colleagues(7). Heparinized blood is centrifuged and the cellular sediment treated with streptolysin O to destroy erythrocytes and polymorphonuclear leukocytes. The remaining cellular debris is concentrated by centrifugation and smeared out on slides for staining by the Papanicolaou technic.

All slides were scanned systematically with a mechanical stage; all positive or equivocal cell forms were marked and restudied until classified.

Results. In no case was a cell remotely resembling ectopic trophoblasts retrieved from the antecubital vein of a pregnant patient. Occasional bizarre forms were observed but rejected as blood cell debris upon close examination. In 3 cases, all sampled at

*Aided in part by USPHS Grants and by the Lydia Raymond Research Fund, Framingham Union Hospital.

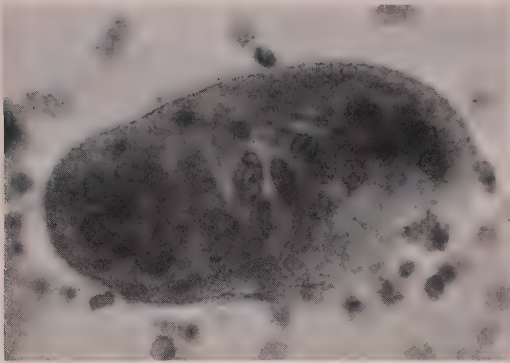


FIG. 1. Embolic syncytial trophoblast recovered from placental site at time of Caesarean section. This syncytial knot exhibits typical multinucleate pattern, peripheral brush border, and abscissional zone. Operation performed because of pelvic disproportion and toxemia. (F.U.H. Case No. 154988.)

the placental site, questionable multinucleate debris was isolated, but distinction between partially disintegrated syncytium and fortuitously coalescent lymphocytic flotsam was not possible.

In a fourth case, a single syncytial embolus was isolated from the placental site (Fig. 1). This cell, evidently a detached knot, was completely consistent with syncytial trophoblast by the usual histological criteria. This patient FUH #154988, a 29-year-old Gravida II Para I, came to Caesarean section because of pelvic disproportion and toxemia.

Discussion. The clearcut identification of embolic trophoblast *ex situ* presents a challenge to even the most experienced observer, particularly in evaluation of severely autolysed remnants. The ubiquitous megakaryocyte, first described as a frequent impactus in the pulmonary capillaries by Aschoff(8), is easily distinguished from trophoblast except in poorly preserved specimens. The isolation of Langhans cells, however, is much more difficult; except in metastatic mole or choriocarcinoma, it is practically impossible to identify them cytologically as emboli, and published reports of cytotrophoblastic embolism must be evaluated with extreme caution. In the more than one thousand slides of the Boston Lying-in Hospital series reported earlier(3), not one definite Langhans cell was found although many hundreds of syncytial emboli were identified. The blood samples

examined in the present series yielded a variety of bizarre cellular elements, but none was felt to warrant identification as trophoblast.

The demonstrations by Douglas and his group(4) are wholly consistent with syncytium, and are valuable documentation of a previously assumed but uncharted stage along the route from chorion to lung. Our own experience in sampling the peripheral circulation has confirmed our *a priori* impression of the near futility of small and random samples in attempting to trap circulatory emboli which occur infrequently and inconstantly. Whether uterine commotion, a classically postulated agent in setting trophoblastic emboli adrift in the maternal circulation, may explain the greater yield in the Douglas series is a matter of speculation.

The absence of trophoblastic cells from antecubital specimens in either study is significant. Most workers have assumed almost total trapping of embolic matter in the pulmonary capillaries. While the possibility of passage through the lung may not be excluded, the phenomenon remains wholly undocumented. Animal studies reported elsewhere(3) have suggested virtually complete filtration of trophoblastic emboli in the lung; follow-up experiments planned in this laboratory will attempt a review under more stringent conditions. Granted the occasional passage of a trophoblastic fragment through the pulmonary capillaries, it is hardly necessary to stress the extreme unlikelihood of retrieving such a seldom traveler with a random venipuncture.

Summary. A total of 155 blood samples from the peripheral circulation of patients in varying stages of pregnancy have been screened for embolic trophoblastic cells by one of the standard technics for segregation of circulating cancer cells. A single syncytial embolus has been found in a specimen from the placental site at time of section; all other samples have been negative. Statistical and technical difficulties are discussed briefly.

1. Schmorl, G. G., *Pathologisch-anatomische Untersuchungen über Puerperal-Eklampsie*. Vogel, Leipzig, 1893.

2. Park, W. W., *J. Path., Bact.*, 1958, v75, 257.
3. Bardawil, W. A., Toy, B. L., *Ann. N. Y. Acad. Sci.*, 1959, v80, 197.
4. Douglas, G. W., Thomas, L., Carr, M., Cullen, N. M., Morris, R., *Am. J. Obst. Gyn.*, 1959, v78, 960.
5. Sandberg, A. A., Moore, G. E., *J. Nat. Cancer Inst.*, 1957, v19, 1.
6. Malmgren, R. A., Pruitt, J. C., Del Vecchio, P. R., Potter, J. F., *ibid.*, v20, 1203.
7. Long, L., Roberts, S., McGrath, R. *J.A.M.A.*, 1959, v170, 1785.
8. Aschoff, L., *Virch. Arch.*, 1893, v134, 11.

Received February 21, 1961. P.S.E.B.M., 1961, v106.

Relation of the Mecholyl Test to Catecholamine Excretion.* (26503)

ARNOLD G. BLUMBERG AND HARRY GOLDENBERG

(With technical assistance of Daniel L. White)

Departments of Internal Medicine and Biochemistry, Hillside Hospital, Glen Oaks, N. Y.

The mecholyl (methacholine) test has been proposed as a measure of sympathetic tone (1). Funkenstein(2) postulated that the differences in reactivity of the systolic blood pressure in human subjects to injection of mecholyl was related to the relative excretion of epinephrine and norepinephrine. He suggested that those subjects whose blood pressures returned rapidly to the base line after injection of mecholyl had reacted by producing more norepinephrine; in contrast, those whose blood pressures stayed low for a long period were assumed to produce more epinephrine in response to stress. Elmadjian (3) studied urinary excretion of epinephrine and norepinephrine and was able to correlate the mecholyl area with the excretion of norepinephrine but not with epinephrine. Manger(4) studied the blood level of epinephrine and norepinephrine and could demonstrate no change in these levels with injection of mecholyl. Since we have been able to standardize the procedure and interpretation of the mecholyl test(5), it seemed appropriate to reinvestigate the relationship between the urinary excretion of the active catecholamines and mecholyl area.

Methods. The mecholyl test was carried out as outlined previously(5). Subjects were patients at Hillside Hospital, a voluntary psychiatric institution. They were seen early in their hospital stay, and were not receiving specific medication at the time of study. In

all, 48 patients were studied. Each was tested under basal conditions. A urine sample was collected prior to the test. The patient then rested for at least a half-hour during which time his blood pressure was automatically recorded at intervals of one minute by a recording sphygmomanometer. When the pressure was noted to be steady, an injection of 1 cc of mecholyl (10 mg) was given subcutaneously. The change in pressure was recorded over the next 20 minutes. A urine sample was then collected. The mecholyl area was determined by plotting systolic pressure *vs.* time in minutes on graph paper (10 squares/cm) and measuring the area enclosed by means of a compensating polar planimeter. The area was corrected for the basal blood pressure as previously described.

For control purposes, the mecholyl test procedure was repeated in 10 selected patients using 1 cc of normal saline (subcutaneous) instead of mecholyl. Blood pressure areas and urine analyses were conducted as for the standard test.

Chemical analyses. Urine specimens (25-50 cc) were analyzed for epinephrine and norepinephrine by the fluorometric method of von Euler and Floding(6). Creatinine content was determined simultaneously, and catecholamine output calculated as $\mu\text{g/g}$ creatinine.

Observations. In the 10 control studies in which saline alone was injected (Table I), there was relatively little change in blood pressure as manifested by areas generally

* This study was supported by a research grant from U.S.P.H.S.

TABLE I. Excretion of Epinephrine and Norepinephrine in Urine Following Injection of Saline (1 cc) and Mecholyl (10 mg).

Pt.	Saline							Mecholyl						
	Area	NE	NE'	NE'/NE	E	E'	E'/E	Area	NE	NE'	NE'/NE	E	E'	E'/E
1	+8.5	35.3	116.0	3.3	2.4	1.3	.5	-20.8	21.3	91.2	4.3	5.9	21.5	3.6
2	+4.5	18.8	19.7	1.0	6.9	12.8	1.9	+71.5	22.1	21.5	1.0	9.1	18.5	2.0
3	-.8	43.0	50.2	1.2	12.1	9.3	.8	-16.4	8.8	27.1	3.1	5.8	7.8	1.3
4	+3.6	59.6	40.8	.7	14.0	19.2	1.4	-28.5	16.0	18.3	1.1	19.2	22.6	1.2
5	-5.5	32.6	67.5	2.1	7.3	3.1	.4	-29.5	16.5	14.0	.9	17.7	26.9	1.5
6	+2.6	12.0	9.5	.8	6.5	2.6	.4	-20.8	9.0	9.2	1.0	1.7	5.0	2.9
7	-2.5	17.8	24.5	1.4	10.3	9.6	.9	+24.8	10.4	35.3	3.4	2.0	10.3	5.1
8	+1.5	16.4	12.5	.8	2.2	2.3	1.0	+9.2	11.6	20.2	1.7	3.4	2.9	.9
9	-14.1	27.0	41.3	1.5	7.3	19.9	2.7	-7.8	75.4	63.9	.8	6.7	6.9	1.0
Avg				1.4			1.1				1.9			2.2

Area = Cm^2 area enclosed by curve of systolic blood pressure for 20 min. after inj. NE = Excretion of norepinephrine before inj. expressed as $\mu\text{g/g}$ creatinine. NE' = Excretion of norepinephrine after inj. expressed as $\mu\text{g/g}$ creatinine. E = Excretion of epinephrine before inj. expressed as $\mu\text{g/g}$ creatinine. E' = Excretion of epinephrine after inj. expressed as $\mu\text{g/g}$ creatinine.

well within the range of $\pm 10 \text{ cm}^2$. Norepinephrine tended to rise after injection of saline to give a post-to-pre excretion ratio of 1.4. Average epinephrine excretion was virtually unaltered (ratio, 1.1). This compares with ratios after mecholyl injection of 1.9 for norepinephrine and 2.2 for epinephrine. The statistical significance of these numbers is relatively low in view of the small number of tests.

Analysis of the entire group of mecholyl tests revealed a fairly consistent increase in norepinephrine and epinephrine excretions. The mean ratio of norepinephrine excretion after mecholyl injection to the basal value was 1.65. The mean ratio for epinephrine excretion was 2.04.

The correlation of the ratio of norepinephrine excretion before and after mecholyl is presented in Fig. 1. While there is a fairly scattered distribution of these values, the Pearson's product moment coefficient has been calculated to be 0.306, with a standard error of ± 0.13 . Values for the ratio of epinephrine excretion before and after mecholyl are graphed against the mecholyl area in Fig. 2. The correlation here is much poorer, with a Pearson's product moment coefficient of 0.101, standard error ± 0.14 .

The correlation of mecholyl area against age was calculated for this group of patients to be 0.486 ± 0.11 , which is significant at the 0.05 level.

Discussion. Armstrong and McMillan(7),

Axelrod(8), and Kirshner *et al.*(9) have demonstrated several 3-O-methylated metabolites in urine which are derived from epinephrine and norepinephrine. It is estimated that about 50% of administered epinephrine is converted to metanephrine (free and sulfate-bound) in humans. 3,4-Dihydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid represent 40% of the residual metabolites. These compounds are not measured by the fluorometric technic employed for catecholamine analysis. The chemical assay of epinephrine and norepinephrine in the urine thus represents only a small fraction, possibly 3%, of the original amounts of these compounds. As a consequence, minor alterations in production of the major metabolites would be expected to incur highly significant changes in the measured excretion of the unaltered catecholamines. Any conclusions drawn from excretion studies of the unchanged hormones must be reviewed in the light of these basic considerations.

The finding of any correlation between the values for norepinephrine and epinephrine excretion and any other parameter must then be considered suggestive. Although the correlation of norepinephrine excretion with the mecholyl area is at the limit of statistical significance, it is not inconsistent with the theoretical assumption that the mecholyl test is an index to sympathetic hyperreactivity, and that norepinephrine excretion levels are in some way related to mecholyl reactivity. The posi-

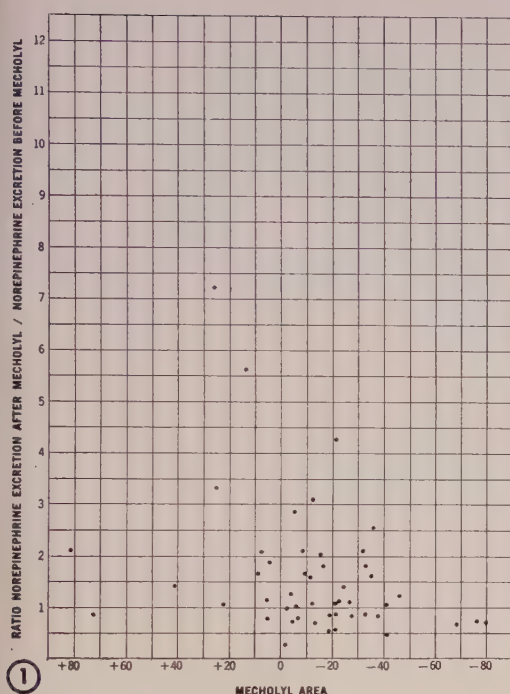


FIG. 1. Relationship of mecholyl area to ratio of norepinephrine excretion.

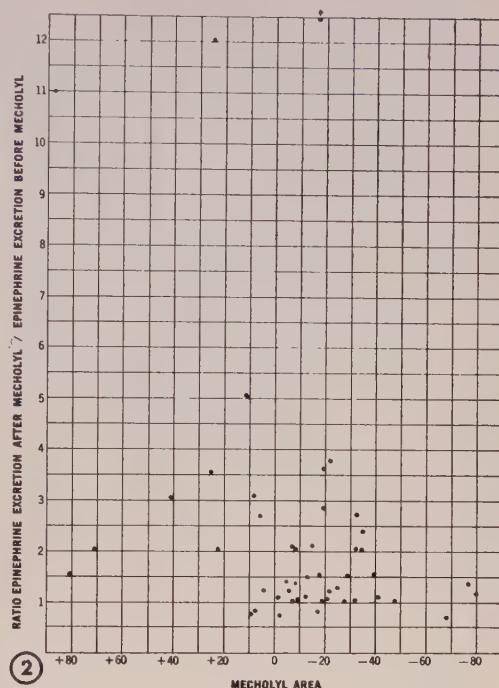


FIG. 2. Relationship of mecholyl area to ratio of epinephrine excretion.

tive (albeit poor) correlation between the trend in the mecholyl area and the norepinephrine excretion ratio also fits in with the concept that higher reactivity of the sympathetic nervous system is related to norepinephrine production. The poorer correlation with epinephrine allows no conclusion to be drawn other than that when mecholyl is given excretion of epinephrine is increased. Correlation coefficients between the epinephrine and norepinephrine ratios and age were poorer than those with the mecholyl area.

The fact that any correlation between mecholyl areas and excretion patterns of epinephrine and norepinephrine exists must be considered significant in view of the relatively small fraction of the amount of these substances produced which can be measured. If the quantity of metanephrine and normetanephrine could be studied, it might be expected to give a more accurate index of production of the pressor substances in response to the mecholyl stress.

Summary. Following injection of mecholyl, excretion of epinephrine and norepinephrine tends to increase. Excretion ratios for nor-

epinephrine and for epinephrine were compared with the relative change in blood pressure after injection of mecholyl. There is a correlation of low statistical significance between norepinephrine excretion ratios and mecholyl area, which suggests that the more hypertensive responses to mecholyl may be associated with higher norepinephrine reactivity.

1. Gellhorn, E., *Physiologic Foundations of Neurology and Psychiatry*, Univ. of Minnesota Press, Minneapolis, 1953.
2. Funkenstein, D. H., Greenblatt, M., Solomon, H. C., *Am. J. Psychiat.*, 1952, v108, 652.
3. Elmadjian, F., Hope, J. M., Freeman, H., *A.M.A. Arch. Neurol. and Psychiat.*, 1957, v77, 399.
4. Manger, W. M., et al., *ibid.*, 1957, v78, 396.
5. Blumberg, A. G., *Psychosomat. Med.*, 1960, v22, 32.
6. von Euler, U. S., Floding, I., *Scand. J. Clin. and Lab. Invest.*, 1956, v8, 288.
7. Armstrong, M. D., McMillan, A., Shaw, K. N. F., *Biochim. et Biophys. Acta*, 1957, v25, 442.
8. Axelrod, J., *Physiol. Rev.*, 1959, v39, 751.
9. Kirshner, N., Goodall, McC., Rosen, L., *Proc. Soc. Exp. Biol. and Med.*, 1958, v98, 627.

Received February 20, 1961. P.S.E.B.M., 1961, v106.

Hypoferremia in Rats Following Injection of Bacterial Endotoxin. (26504)

RALPH F. KAMPSCHMIDT AND GLORIA A. SCHULTZ

Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Okla.

The anemia of infection has been shown to be associated with or accompanied by a hypoferremia(1). Both the anemia and hypoferremia were produced by several different bacteria and also by turpentine, but endotoxins from bacteria were found to be relatively ineffective(2). Ho and Kass, however, showed that a mild hemolytic anemia could be produced in rats by injections of bacterial endotoxins(3). In the experiments by Cartwright *et al.*(2) daily injections of *Staphylococcus* toxin for several days produced only a moderate lowering of plasma iron and typhoid vaccine was without effect. It has also been shown that daily injections for several days will result in development of a tolerance to several other effects of bacterial endotoxins(4).

The experiments reported here demonstrated that a single injection of a small amount of bacterial endotoxin caused a marked lowering of plasma iron, and that a tolerance was developed with daily injections.

Methods. Female Holtzman rats 50 to 70 days old and weighing 170-200 g were used in these experiments. Lipopolysaccharides from *Escherichia coli* 055:B5, *Serratia marcescens* and *Staphylococcus aureus* were obtained from Difco Laboratories, Detroit, Mich. These lipopolysaccharides were dispersed in pyrogen-free distilled water, and 1 ml was injected intraperitoneally into each rat. Plasma iron was measured by the method of Schade *et al.*(5).

Results. Plasma iron was markedly decreased by all 3 lipopolysaccharides with a maximum depression occurring approximately 16 hours after a single injection. The plasma iron concentration of rats receiving 3 different doses of these endotoxins is shown in Table I. The lipopolysaccharide from *E. coli* caused the greatest lowering of plasma iron concentration, but all produced a significant hypoferremia in doses as low as 0.1 μ g per rat.

Two days after administration of 1 μ g of endotoxin plasma iron returned to the normal value (Table II). Determination of plasma iron 16 hours after last injection, in a series of 4 daily injections, indicated development of a tolerance with repeated injections.

Discussion. Studies by Janoff, Zweifach and Shapiro(6) on the levels of plasma iron in various types of experimental shock indicated a decreased concentration after administration of lethal doses of *E. coli* endotoxin. The degree of hypoferremia due to endotoxin was difficult to evaluate due to the decrease of plasma iron in control animals under their experimental conditions.

It appears quite likely that failure of Cartwright *et al.*(2) to observe a marked hypoferremia after administration of endotoxins was due to the length of time between injection and measurement of plasma iron, as well as development of a tolerance after daily injection.

A similar observation of a decrease in plasma iron after a single injection, and development of a tolerance after daily injection has been observed with extracts prepared from tumor tissues(7). It was found possible to overcome this tolerance by frequent administration of small amounts of material

TABLE I. Plasma Iron Concentration in the Rat 16 Hours after Intraperitoneal Injection of Endotoxin.

Source	Dose (μ g/rat)	No. of rats	Bound plasma iron (μ g %)
Sham	0	39	238 \pm 6*
<i>Escherichia coli</i> 055:B5†	10	9	50 \pm 8
	1	15	82 \pm 7
	.1	9	116 \pm 12
<i>Serratia marcescens</i> †	10	6	103 \pm 5
	1	6	131 \pm 9
	.1	6	156 \pm 13
<i>Staphylococcus aureus</i> †	10	6	91 \pm 6
	1	6	150 \pm 10
	.1	6	179 \pm 14

* Mean \pm stand. error.

† Obtained from Difco Labs, Detroit, Mich.

TABLE II. Comparison of Effects of Single and Repeated Injections of 1 μ g of *E. coli* Lipopolysaccharide on Plasma Iron in the Rat.

No. and frequency of injections	Time since last inj. (hr)	No. of rats	Bound plasma iron (μ g %)
1 Sham*	16	39	238 \pm 6†
1	16	15	82 \pm 7
1	48	3	280 \pm 46
1	72	3	217 \pm 18
4 daily	16	6	235 \pm 15

* One ml of pyrogen-free distilled water.

† Mean \pm stand. error.

at injection sites where it would be absorbed slowly(8).

Summary. A single injection of as little as 0.1 μ g of lipopolysaccharide from bacteria produced a marked hypoferremia in the rat. A tolerance was developed with daily administration of the endotoxin.

1. Cartwright, G. E., Wintrobe, M. M., in *Advances in Internal Medicine*, Chicago, Year Book Publishers, 1952, p165.

2. Cartwright, G. E., Lauritsen, M. A., Humphreys, S., Jones, P. J., Merrill, I. M., Wintrobe, M. M., *J. Clin. Invest.*, 1946, v25, 81.

3. Ho, M., Kass, E. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1958, v97, 505.

4. Atkins, E., *Physiol. Rev.*, 1960, v40, 580.

5. Schade, A. L., Oyama, J., Reinhart, R. W., Miller, J. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1954, v87, 443.

6. Janoff, A., Zweifach, B. W., Shapiro, L. R., *Am. J. Physiol.*, 1960, v198, 1161.

7. Kampschmidt, R. F., Adams, M. E., McCoy, T. A., *Cancer Research*, 1959, v19, 236.

8. Kampschmidt, R. F., Clabaugh, W. A., *J. Nat. Cancer Inst.*, 1960, v25, 713.

Received February 23, 1961. P.S.E.B.M., 1961, v106.

Effects of Exposure to Continuous Light on the Eye of the Growing Chick.* (26505)

J. K. LAUBER, J. V. SHUTZE AND J. MCGINNIS

Department of Poultry Science, Washington State University, Pullman

A peculiar eye abnormality observed in chicks exposed to continuous light during the first 6 weeks of life has been briefly described (1). This observation has been confirmed repeatedly, using several different breeds of chicks(2). Further work has been conducted on this abnormality to obtain information on the histological changes associated with the eye enlargement.

Day-old, male chicks were randomly placed in floor pens from which natural light was excluded. Incandescent bulbs were used to provide approximately 3-foot-candle intensity measured at bird height. The chicks were brooded under shielded electric brooders in the normal manner. Feed and water were supplied *ad libitum*.

One group of chicks was exposed to 12 hours light per day (controls) and another group received 24 hours light per day. The

birds exposed to 24 hours light per day invariably developed elongated eyelids, or a peculiar "slant eyed" condition. The cornea appeared to be less convex than in controls, with the iris closely associated with the inner surface of the cornea. Fig. 1 illustrates these effects.

Four birds, 2 from each treatment, were sacrificed at 10 weeks of age. The eyeballs were removed, trimmed of external musculature and weighed (Table I). The average weights agree with previous reports based on a much larger number of birds(1,2). The eyeballs were then fixed in Bouin's solution for 24 hours, cut in half with a razor blade and kept in fixative for 24 hours more. A comparison between 2 such longitudinally sectioned eyes (Fig. 2), shows flattening of the cornea and reduction in thickness of the eye wall. The most striking difference appears to be in size of the vitreous body. The hemispheres were embedded in paraffin, sec-

* Scientific Paper No. 2087, Washington Agri. Exp. Stations, Pullman. Project No. 1444.



FIG. 1. Effect of continuous incandescent light (left) compared with 12 hr light/day (right) on appearance of eyes of chicks.

tioned at 10 μ , and examined histologically.

A distinct difference was noted in total thickness of the wall of the eye, as well as in the thickness of some of its layers. In some areas, the choroid appeared to be somewhat thinner in the eyes of birds exposed to

TABLE I. Influence of Light Exposure of Chicks on Size of Eyes.

Light exposure/day (hr)	Wt of eyes	
	g	As % of body wt
24	6.93	.456
12	5.21	.377

continuous light, the vessels being flattened and elongated when compared with those in the eyes of controls. Differences in thickness

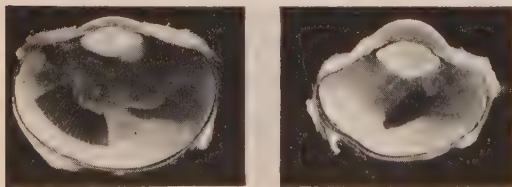


FIG. 2. Comparison of longitudinal sections of eyeballs from chicks exposed to continuous light (left) and to 12 hr light/day (right).

of the retina were most pronounced: the retina of the birds which received continuous light exposure was markedly thinner (Fig. 3). The thickness of the nerve fiber layer and the layer of rods and cones appeared to be greatly reduced in chicks exposed to continuous light. The pigmented layer, and the inner nuclear layer appeared to be slightly thicker in the experimental birds which had thinner retinas. No differences could be observed in the optic nerve or the pecten after staining with either hematoxylin and eosin or with Masson's trichrome method.

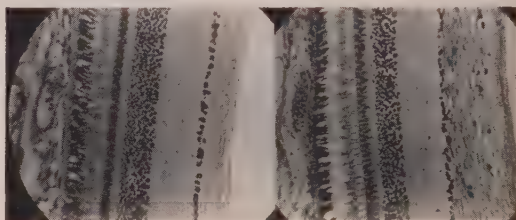


FIG. 3. Photomicrographs of retinas from chicks exposed to continuous light (left) and to 12 hr light/day (right). Mag. 115 \times .

Summary. The influence of continuous incandescent light on the eyes of young chicks has been investigated and a profound enlargement of the eyeball has been observed. The enlargement is associated with an accumulation of fluid in the vitreous body. Histological changes were evident in several layers of the eye with the most striking effect being a thinning of the retina. Exposure to continuous light reduced the thickness of the nerve fiber layer and the layer of rods and cones.

1. Jensen, L. S., Matson, W. E., *Science*, 1955, v125, 741.

2. Shutze, J. V., Jensen, L. S., Carver, J. S., Matson, W. E., *Washington Exp. Sta. Tech. Bull.*, No. 36, 1960.

Received February 24, 1961. P.S.E.B.M., 1961, v106.

Thyroxine and Mammary Gland Growth in Rat.* (26506)

DAVID R. GRIFFITH[†] AND CHARLES W. TURNER

(With technical assistance of Mary E. Powell)

Dept. of Dairy Husbandry, University of Missouri, Columbia

Previous studies in this laboratory have employed desoxyribonucleic acid (DNA) as a quantitative index of mammary gland cellular proliferation in rats and mice(1,2,3). By this method, it is possible to determine the synergistic influence of various hormones on normal and experimental mammary gland growth.

Grovesnor and Turner(4,5) reported administration of hormones concerned with lactation in optimal amounts increased milk yield and decreased variation among animals. Similarly, suboptimal secretion of hormones concerned with mammary gland cellular proliferation may also limit maximum growth of gland.

With elaboration of quantitative measures of mammary gland growth and thyroxine secretion rate (TSR)(5,6), it seemed desirable to investigate influence of physiological levels of exogenous thyroxine on mammary gland proliferation of normal pregnant rats.

Methods. Groups of pregnant rats of Sprague-Dawley-Rolfsmeyer strain were used. Control animals were sacrificed on days 18-20 of pregnancy and experimental groups 20 days after detection of sperm within vagina. Experimental animals were injected with 2.5, 3.0, and 3.5 μg of thyroxine (T_4)/100 g body weight (BW) respectively from days 3 to 19 of gestation. Six abdominal-inquinal mammary glands were removed from each rat and DNA determined as described previously(3).

Results. Mean total DNA of normal pregnant rats was 7.63 mg/100 g BW; whereas experimental groups were 9.38, 8.09, and 9.6 mg/100 g BW respectively for 2.5, 3.0, and 3.5 μg of T_4 /100 g BW (Table I). DNA/mg followed a similar pattern: normal (33.2 μg), 2.5 μg T_4 (38.3 μg), 3.0 T_4 (31.8 μg),

and 3.5 μg T_4 (38.3 μg). DNA/100 g BW was significantly increased at 2.5 and 3.5 T_4 levels ($P>.01$) when compared with normal pregnant values. There was a slight but non-significant increase in DNA/100 g at 3.0 μg T_4 level and also a slight decline in DNA/mg. Combined T_4 total DNA was also increased significantly ($P>.01$) compared to control pregnant animals. Calculation of frequency distribution of normal and T_4 injected pregnant animals (Fig. 1) indicated that T_4 increased mean total DNA; whereas range and variability of total DNA remained essentially the same between the 2 groups. DFFT of all groups of animals remained constant, and increase in total DNA was due to an increase in DNA/mg DFFT.

Discussion. Past studies of thyroid-mammary gland relationship were complicated by administration of unphysiological levels of T_4 . It is now possible to measure thyroid activity of individual animals and variation in secretion rate by TSR method(5,6). This procedure enables one to determine effect of physiological levels of T_4 on mammary gland development.

Grovesnor and Turner(5) reported mean daily TSR of non-lactating rats was 1.3 μg /100 g/day, while mean value of lactating animals was 2.2 μg /100 g/day. The highest observed value was 3.0 μg /100 g/day.

Studies(7,8) have indicated T_4 is not essential for experimental development of rat mammary glands. Chen *et al.*(7) reported triply-operated rats appeared to have extensive mammary gland development with evidence of lactation without addition of T_4 to the injected hormonal regime. In this laboratory(8), addition of 3 μg T_4 /100 g BW with 2 μg estradiol benzoate (EB) and 6 mg progesterone (P) had little beneficial effect in increasing total DNA of rat mammary glands in thyro-parathyroidectomized animals. Although DNA/mg DFFT was increased, total DNA remained the same due

* Contribution from Mo. Agr. Exp. Sta., Journal Series No. 2258. Approved by the Director.

[†] Post doctoral Research Fellow of Nat. Cancer Inst. This investigation supported in part by grants from the U.S.P.H.S. and Am. Cancer Soc.

TABLE I. Thyroxine and Mammary Gland Growth in Pregnant Rats.

No. of animals	Stage of gestation (days)	Thyroxine level*	Body wt† (mean)	DFFT (mg) mean	DNA ($\mu\text{g}/\text{mg}$ DFFT) mean \pm S.E.	Total DNA (mg/100 g B.W.) mean \pm S.E.
19	18-20	—	324	749	33.24 \pm .8	7.63 \pm .39
17	20	2.5	280	724	38.3 \pm 2.28	9.38 \pm .32 ^{1,2}
13	20	3.0	287	745	31.8 \pm 4.9	8.09 \pm 1.29
15	20	3.5	283	740	37.7 \pm 1.43	9.66 \pm .46 ^{1,2}
45‡	20	2.5, 3.0 & 3.5	283	736	35.9 \pm 1.44	9.07 \pm .25 ^{1,2}

* Corrected for wt of fetuses and uterus.

† μg T_4 /100 g/day from day 3-19 of pregnancy.‡ Aggregate T_4 animals.¹ Significant to normal pregnant DNA values at 1% level.² Significant to 3.0 T_4 DNA at 2% level.

to a significant reduction in DFFT/100 g.

Moon and Turner(9) determined effect of mild hyperthyroidism upon experimental development of rat mammary glands using DNA as an index of growth. Injection of 3 or 6 μg T_4 /100 g BW with 1 μg EB and 3 mg P/day did not significantly increase mammary gland growth above animals which received EB and P alone. When amount of EB and P was doubled, a significant increase in total DNA with this level of T_4 was observed (26%). It was suggested by the authors suboptimal secretion of T_4 may be one of the limiting factors in obtaining maximum mammary gland proliferation.

In the present study, levels of T_4 were employed which approximated average TSR of lactating animals (2.5 $\mu\text{g}/100$ g/day), the highest TSR obtained during lactation (3.0 $\mu\text{g}/100$ g/day) and a level slightly greater than observed (3.5 $\mu\text{g}/100$ g/day).

Data obtained indicate that mild hyperthyroidism is beneficial in stimulating increased growth of rat mammary glands during pregnancy. The group of 45 pregnant animals injected with T_4 showed a mean

DNA of $9.07 \pm .25$, an increase of 22% above normal pregnant rats. Significantly greater DNA obtained with T_4 was not due to an increase in DFFT. As indicated by DNA/mg, cell concentration/mg tissue accounted for the increase in total DNA at the 2.5 and 3.5 μg level of T_4 .

Summary. The effect of mild hyperthyroidism upon mammary gland growth has been studied by injection of exogenous T_4 into normal pregnant rats at levels of 2.5, 3.0, and 3.5 $\mu\text{g}/100$ g BW/day. Normal pregnant rats showed mean total of DNA of $7.63 \pm .39$ mg/100 g BW. Group of 45 pregnant rats injected with T_4 showed a mean DNA of $9.07 \pm .25$ mg/100 g BW, an increase of 22%. It is suggested sub-optimal T_4 secretion may be one of the limiting factors in attainment of maximum mammary gland proliferation.

1. Moon, R. C., Griffith, D. R., Turner, C. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v101, 788.
2. Brookreson, A. D., Turner, C. W., *ibid.*, 1959, v102, 744.
3. Griffith, D. R., Turner, C. W., *ibid.*, 1959, v102, 619.
4. Grovesnor, C. E., Turner, C. W., *ibid.*, 1958, v99, 517.
5. ———, *ibid.*, 1959, v100, 162.
6. Pipes, G. W., Premachandra, B. N., Turner, C. W., *J. Dairy Sci.*, 1957, v40, 340.
7. Chen, T. T., Johnson, R. E., Lyons, W. R., Li, C. H., Cole, R. D., *Endocrinol.*, 1955, v57, 153.
8. Von Berswordt-Wallrabe, R., Turner, C. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v103, 536.
9. Moon, R. C., Turner, C. W., *ibid.*, 1960, v103, 149.

Received February 27, 1961. P.S.E.B.M., 1961, v106.

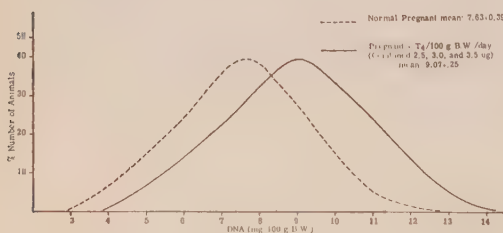


FIG. 1. Frequency distribution of DNA of mammary glands of normal rats at end of pregnancy compared with similar rats inj. with thyroxine during pregnancy. Thyroxine increased gland growth 22%.

Acquired Immunological Tolerance of Male Skin Isografts in Adult Female Mice Injected with Whole Blood.* (26507)

HARVEY KELMAN, CARLOS MARTINEZ,[†] AND ROBERT A. GOOD[‡]

Departments of Physiology and Pediatrics, University of Minnesota Medical School, Minneapolis

Lasting immunological tolerance of skin homografts in animals injected with whole blood from the prospective donors has been obtained during fetal life in chicks(1) or during the neonatal period in chicks(2), rats(3) and dogs(4). More recently Billingham and Silvers(5) have obtained permanent tolerance of male skin isografts in female mice of the C57 Bl/6 strain injected intraperitoneally at birth with various concentrations of peripheral blood leucocytes taken from male donors. However, attempts to establish tolerance of homologous skin grafts in weanling and adult rabbits or rats by injecting these animals with whole blood taken from the prospective donors have resulted only in a significant delay in survival time of subsequent homologous grafts(6,7).

On the basis of these results and certain theoretical considerations we have decided to investigate further the possibility of inducing permanent tolerance of male skin isograft in adult female mice of the C57Bl/1 strain previously transfused with different amounts of whole blood taken from mature male donors of the same strain. Female mice of this strain regularly reject skin grafts taken from isologous males although the weak histocompatibility difference between donor male and recipient female allows induction of tolerance in the females to the male graft by exposure to male antigens in the form of viable spleen cells during the neonatal period as well as in adult life(8).

The experiments reported herein demonstrate that lasting tolerance of male skin isografts can, indeed, be induced in adult female mice of the C57Bl/1 strain by pretreatment of these animals with whole blood taken from isologous mature male donors.

Method. Adult male and female mice of the C57Bl/1 strain were used. Pooled whole blood obtained by decapitation under ether anesthesia of stock C57Bl/1 adult male mice was collected in a test tube containing a minimal amount of heparin solution.[§] This blood was immediately injected intravenously into the lateral subcutaneous vein of the tail of several groups of isologous females 60 days of age. Recipient mice were divided into groups according to the amount of blood transfused, as follows: 0.1 cc, 0.25 cc, 0.50 cc, 0.75 cc and 1 cc of whole heparinized blood. Control groups consisting of untreated 60 day old C57Bl/1 female mice and mice receiving 0.75 cc of isologous female whole blood were also prepared.

Three to 4 days after blood transfusion, pretreated and untreated female mice received a full-thickness abdominal skin graft taken from male donors of the same strain. The technic of skin grafting as well as the criteria used to determine the success or failure of the graft was the same as that previously described(9).

Mice were housed individually in plastic cages and fed Purina Laboratory Chow and tap water. Grafted animals were observed for at least 5 months and only those animals showing a well established graft growing copious hair in a direction opposite to that on the skin of the host were judged to be tolerant of male skin isograft.

Results. The results are summarized in Table I. Of the group of untreated C57Bl/1 female mice grafted with male skin 4 out of 19 (21%) accepted this graft. Likewise, of the control group consisting of C57Bl/1 female mice injected with 0.75 cc of isologous female whole blood 2 out of 12 (17%) accepted the male skin isograft. On the other hand, of the females pretreated with isologous whole blood taken from male donors each of

* Aided by grants from the Am. Cancer Soc. and USPHS.

[†] Amer. Cancer Soc., Prof. of Physiology.

[‡] Amer. Legion Memorial Heart, Prof. of Pediatrics.

[§] Liquaemin Sodium, Organon, Inc.

TABLE I. Induction of Lasting Immunological Tolerance of Male Skin Isograft in Adult Female Mice of C57Bl/1 Strain Injected Intravenously with Various Amounts of Male Whole Blood.

Host strain	Skin donor strain	Pretreatment, whole blood cc/mouse	Sex	No. females accepting male skin isografts/ No. animals grafted
C57Bl/1 ♀	C57Bl/1 ♂	.10	♂	3/15
<i>Idem</i>	<i>Idem</i>	.25	♂	5/10
"	"	.50	♂	12/14
"	"	.75	♂	10/12
"	"	1.00	♂	5/7
"	"	.75	♀	2/12
"	"	None		4/19

the groups except that in which 0.1 cc of blood was injected showed a significant increase in male skin isograft acceptance as compared to that observed in groups of control animals. In the group injected with 0.10 cc 3 out of 15 treated females accepted skin isografts while in the 0.25 cc, 0.50 cc, 0.75 cc and 1.00 cc treated groups respectively 5 of 10, 12 of 14, 10 of 12 and 5 of 7 females accepted male skin isografts.

Discussion. These results are of interest for several reasons. First, they confirm our previous studies in demonstrating that acquired immunological tolerance can be induced in adult female mice of the C57Bl/1 strain by pretreating these animals with male spleen cells injected intravenously in large number or by placing the females in celomic parabiosis with isologous males(8). In the experiments reported herein tolerance of male skin isograft was obtained in adult females injected intravenously with various amounts of peripheral blood taken from isologous male donors. However, establishment of tolerance by this method appears to be related to the amount of male blood injected. For example, while 0.10 cc of fresh whole blood was not sufficient to bring about immunological tolerance of male skin isograft, doses ranging from 0.25 to 1.00 cc induced permanent tolerance in many instances. The difference in incidence of male skin acceptance observed between controls and all experimental groups injected with at least 0.25 cc of blood is statistically significant at the 1% level. The fact that establishment of tolerance by in-

jecting whole blood is related to the dose of blood administered suggests that a minimum amount of blood must be used to produce tolerance, beyond which greater amounts have no appreciable advantage.

Second, our results demonstrate the possibility of inducing immunological tolerance in adult individuals using peripheral blood cells as the preconditioning material. Billingham *et al.*(2) have demonstrated that while whole blood was successful to a small degree as pretreating material of fetal mice to induce this phenomenon, lymphoid tissue was far more efficacious. However, this advantage of lymphoid tissue over whole blood was not observed by Puza and Gombos(4) who gave whole blood to neonatal dogs by the intravenous route.

One question explicit in these results is: Which peripheral blood cells are responsible for establishing immunological tolerance? The smallest dose of whole blood required to produce satisfactory degree of tolerance was 0.25 cc which represents approximately 1,500,000 viable white blood cells. This is less than the number of spleen cells necessary for induction of tolerance in adult mice (8). Yet many of the peripheral cells are end stage elements considered incapable of replication. It could be that the red blood cells themselves play some assistive role in inducing in the recipient animal a state similar to that of immunological paralysis(10) thus allowing time to the injected cells to establish themselves and perhaps replicate to reach a sufficient number to abolish the homograft reaction. We are now investigating this hypothesis.

It seems clear that immunological tolerance of male skin isografts can be produced in adult female mice of the C57Bl/1 strain by pretreatment of these mice intravenously with various amounts of fresh whole blood taken from mature isologous male donors.

Summary. Permanent acquired immunological tolerance has been established in adult C57Bl/1 female mice to isologous male skin graft using pooled whole blood obtained from C57Bl/1 males as a pre-conditioning material. Tolerance using this technic is a dose related

phenomenon. Doses of 0.25 cc of whole blood or greater produce a long lasting tolerance.

1. Billingham, R. E., Brent, L., Medawar, P. B., *Nature*, 1953, v172, 603.
2. ———, *Phil. Trans.*, 1956, vB, 239, 357.
3. Ashley, F. L., Stein, H., Peterson, R., Grazer, F., Longmire, W. P., *Transpl. Bull.*, 1958, v5, 29.
4. Puza, A., Gombos, A., *ibid.*, 1958, v5, 30.
5. Billingham, R. E., Silvers, W. K., *J. Immunol.*, 1960, v85, 14.

6. Stark, R. B., Brownlee, H., Grunwald, R. D., *Ann. N. Y. Acad. Sci.*, 1958, v73, 772.
7. Stark, R. B., Dwyer, E., *Surgery*, 1960, v46, 277.
8. Mariani, T., Martinez, C., Smith, J. M., Good, R. A., *Ann. N. Y. Acad. Sci.*, 1960, v87, 93.
9. Martinez, C., Smith, J. M., Aust, J. B., Good, R. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1958, v97, 736.
10. Felton, L. D., *J. Immunol.*, 1949, v61, 107.

Received February 27, 1961. P.S.E.B.M., 1961, v106.

Effect of Reserpine on Peptic Ulceration and Gastric Blood Flow in Dogs.* (26508)

DEMETRE M. NICOLOFF, NELSON H. STONE, ARNOLD S. LEONARD,
RAYMOND DOBERNECK AND OWEN H. WANGENSTEEN

Department of Surgery, University of Minnesota Medical School, Minneapolis

The clinical use of rauwolfia alkaloids has found wide acceptance since their recent introduction to this country mainly for treatment of hypertension and certain anxiety states. However, as with many drugs, these compounds produce undesirable side effects on other physiological systems. Duodenal ulcer, hemorrhage, and perforation have been reported in patients treated with reserpine(1,2). It is not known whether patients treated with rauwolfia drugs develop gastric complications as a result of therapy, because of their disease process and emotional constitution, or a combination of these factors. We attempt in this study to elucidate some of the effects of reserpine on gastric physiology and peptic ulceration in experimental animals.

Peptic ulceration does not occur spontaneously in either dogs or cats. However, ulceration can be produced in laboratory animals by administration of histamine in beeswax(3). This method has proven useful in evaluation of surgical procedures and medications on peptic ulcer diathesis.

The first part of this study demonstrates the effect of reserpine on histamine-induced ulceration in dogs and cats. The second por-

tion shows the effect of reserpine on gastric blood flow.

Method. A. Ulcer provocation. Adult mongrel dogs and cats were used in these experiments. The histamine in beeswax mixture was prepared as described by Code and Varco(3). The dogs were kept in separate cages and the cats were kept 4 to a cage. All animals received food and water *ad libitum*. Although the exact amount of food ingested was not measured, the animals receiving reserpine consumed less of the rations offered.

The animals were divided into 6 groups. Group I (9 dogs) received 30 mg of histamine in beeswax I.M. daily. Group II, (8 dogs) received 1 mg of reserpine I.M. in addition to the histamine. Group III, (8 dogs) received only 1 mg reserpine daily. Group IV (8 cats) received 15 mg histamine in beeswax daily. Group V (8 cats) received 0.5 mg of reserpine in addition to the histamine. Group VI (11 cats) received 0.5 mg reserpine only.

Each animal was injected with histamine in beeswax and/or reserpine intramuscularly at the same time each day. The dosage of reserpine used caused miosis, diarrhea, and lethargy in both dogs and cats. At time of death or sacrifice, the stomach and duodenum of all animals were examined grossly for erosion or ulceration. For the purpose of this

* This work was supported by Peptic Ulcer Grant and Jay and Rose Phillips and Donald J. Cowling Funds for Surgical Research.

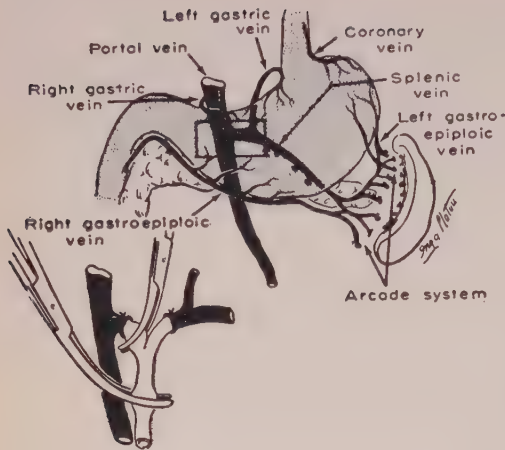


FIG. 1. Diagram of blood flow preparation used.

study "average time of ulceration" is average time of survival of those dogs that died with peptic ulcers. If any doubt existed as to their presence, microscopic sections of questionable areas were prepared and examined.

B. Blood flow studies. In the second portion of our experiment, mongrel dogs weighing between 10 and 15 kg were used. The animals were anesthetized with sodium pentobarbital, 30 mg/kg. The carotid artery was cannulated and arterial pressures recorded *via* a Satham transducer on a Sanborn Polyviso recorder. The abdomen was opened and the right gastric, gastroduodenal and left gastric epiploic veins were ligated and divided. Splenectomy and omentectomy were done (Fig. 1). The left gastric vein was cannulated with a polyethylene catheter which was then connected to a Y connector. A polyethylene catheter which was passed into the portal vein *via* the splenic vein was attached to the other limb of the Y connector. In this way, normal gastric flow could be maintained between measurements and gastric venous outflow could be measured by occlusion of the

portal vein catheter and collection from the common limb of the Y. The dog was given 2-3 mg heparin/kg. Heparinized saline was used to irrigate the catheters. Following cannulation of the portal and left gastric veins, the dog was allowed to stabilize for 30-45 minutes.

Control values for venous outflow were established by at least 2 measurements at $\frac{1}{2}$ hour intervals prior to administration of the drug. The reserpine was then given *via* the carotid catheter and venous outflow was measured at least 3 times at hourly intervals. There were 3 groups of dogs in this part of the experiment. Group I received no medication; Group II, 1 mg reserpine, Group III, 2.5 mg reserpine.

Results. A. Ulcer provocation. It is evident from Table I that reserpine greatly abets histamine ulceration. In fact, reserpine alone appears to be as effective an agent as histamine for production of peptic ulceration and erosions. Reserpine alone caused 50% of dogs to ulcerate in an average time of 6.5 days. The results in cats followed a similar pattern (Table II).

Another interesting finding was that when only histamine was given, ulceration occurred predominantly in the duodenum. However, when reserpine was given alone or with histamine, ulcerations occurred predominantly in the antral and fundic portions of the stomach (Fig. 2 and 3).

All the animals that died with ulceration showed evidence of weight loss, probably due to the decreased intake of food that occurs concomitantly with peptic ulceration. As stated previously, the dogs that received reserpine ate less and this may be a factor in the abatement of histamine induced ulcer.

Baronofsky has shown that nitroglycerine

TABLE I. Dogs.

Group	No. of dogs	Medication	ulceration (%)	Avg time of ulceration, days (\pm S.E.)*	Duration of survival, days	Type of ulcer
I	9	30 mg histamine in beeswax	44	23.0 ± 9.8	2-40	All duodenal ulcers
II	8	<i>Idem</i> + 1.0 mg reserpine	100	$2.4 \pm .4$	1-4	" gastric "
III	8	1.0 mg reserpine	50	6.5 ± 2.6	4-15	" " "

* p value for difference of all means = $<.01$.

TABLE II. Cats.

Group	No. of cats	Medication	ulceration (%)	Avg time of ulceration, days (\pm S.E.)*	Duration of survival, days	Type of ulcer
IV	8	15 mg histamine in beeswax	62.5	9.0 \pm .9	3-12	All duodenal ulcers
V	8	<i>Idem</i> + .5 mg reserpine	89	2.1 \pm .3	1-5	" gastric "
VI	11	.5 mg reserpine	63	4.6 \pm .4	3-8	2 duodenal, 5 gastric ulcers

* p value for difference of all means = $<.01$.

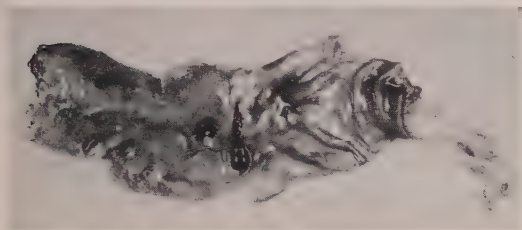


FIG. 2. Ulcer in dog.

and pitressin also abet histamine ulceration (4,5). He suggested that this was due to lowered resistance of the tissues, secondary to ischemia. Recent work has shown that reserpine sensitizes the arterioles in the splanchnic bed to circulating catechol amines(6). The occurrence of gastric rather than duodenal ulceration with reserpine administration stimulated our interest in the effect of reserpine on blood flow in the stomach.

B. Blood flow studies. In Group I, the control group, there was very little change in consecutive blood flow measurements (Table III). In Group II there was a slight rise in blood flow, of dubious significance. In Group III, there is a definite decrease in gastric blood flow following administration of 2.5 mg of reserpine. During all these determinations, blood pressure variations did not exceed 15-25 mm Hg.

Discussion. Under these experimental conditions reserpine greatly enhances histamine induced ulceration. Furthermore, reserpine is just as potent as histamine in induction of

experimental peptic ulceration. This is in accord with the findings of others that reserpine, like histamine, evokes maximal gastric secretion in patients or Heidenhain pouch dogs(7).

Work done in our laboratory has shown that pretreatment of cats with reserpine renders the cat's esophagus much more susceptible to digestion by perfused human gastric juice(8). Determination of blood flow by the K^{42} method suggests that this increased susceptibility of the esophagus may be due to the decreased blood flow in that organ(9).

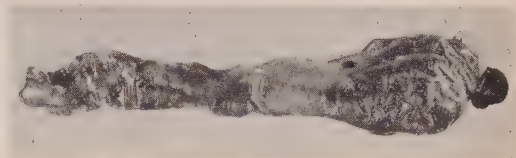


FIG. 3. Ulcer in cat.

Reserpine then could be detrimental to the stomach in 2 ways. In moderate doses it causes augmented acid secretion. In larger doses it causes a decrease in gastric blood flow with resultant ischemia and increased susceptibility to ulceration. There would appear to be, therefore, good evidence to suggest avoidance of rauwolfia derivatives in patients with the peptic ulcer diathesis.

Summary. Reserpine, in large doses, abets histamine induced ulceration in dogs. It also appears to be as ulcerogenic as histamine. Decreased blood flow occurs in the stomach

TABLE III. Effect of Reserpine on Gastric Blood Flow.

Medication	No. of dogs	Mean change in flow (ml) \pm stand. error			
		1 hr	2 hr	3 hr	4 hr
None	6	-3.3 \pm 2.1	-2.7 \pm 1.4	-2 \pm 1.1	2.5 \pm 2.7
1.0 mg reserpine	4	3.3 \pm 1.9	4.3 \pm 2.2	5.8 \pm 4.5	4.0 \pm 2.6
2.5 mg "	5	-19 \pm 3.7	-20 \pm 3.0	-21 \pm 3.6	-19 \pm 2.3

when 2.5 mg of reserpine is given intravenously to dogs. This may be an additional mechanism for the increased incidence of ulceration.

1. Denny, J. L., Frasher, W. G., Hoytt, D. D., *Ann. J. Med. Sci.*, 1955, v230, 169.
2. Hallister, L. E., Krieger, G. E., Krinzel, A., Roberts, R. H., *Ann. N. Y. Acad. Sci.*, 1955, v61, 92.
3. Hay, L. J., Varco, R. L., Code, C. F., Wangenstein, O. H., *S. G. O.*, 1942, v75, 170.
4. Baronofsky, I. D., Wangenstein, O. H., *Bull. Am. Coll. Surg.*, 1945.

5. ———, *PROC. SOC. EXP. BIOL. AND MED.*, 1964, v62, 127.
6. Burns, J. H., Rand, M. I., *Brit. J. Anesthesia*, 1958.
7. Rider, J. A., Swader, J., Gibbs, J. C., Derooin, J., Agcaoili, L., Moeller, H. C., *Clin. Res. Proc.*, 1957, v5, 39.
8. Leonard, A. S., Griffen, W. O., Jr., Wangenstein, O. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v103, 190.
9. Leonard, A. S., Griffen, W. O., Jr., Nicoloff, D. M., Doberneck, R. C., Wangenstein, O. H., *ibid.*, 1961, in press.

Received January 24, 1961. P.S.E.B.M., 1961, v106.

Use of Fungizone® in Control of Fungi and Yeasts in Tissue Culture. (26509)

D. PERLMAN, NANCY A. GIUFFRE AND SHARON A. BRINDLE
(Introduced by R. Donovan)

Squibb Institute for Medical Research, New Brunswick, N. J.

Fungal and yeast contamination is a troublesome problem to those working in tissue culture laboratories. Mycostatin (Squibb Nystatin), an antifungal antibiotic derived from *Streptomyces noursei* was found by McLimans *et al.*(1) to have little or no effect on growth of mammalian cells and to suppress growth of fungi and yeasts effectively. As it is relatively insoluble in aqueous media, it has been difficult to use in certain tissue culture systems, especially where the cells are being grown in monolayers. Fungizone®*, the sodium deoxycholate complex of amphotericin B(2) an antifungal agent produced by *Streptomyces nodosus*(3), forms a colloidal dispersion in aqueous media and is effective in suppressing growth of many yeasts and fungi(4). Hemphill *et al.*(5) found that Fungizone was effective in controlling growth of certain yeasts and fungi in tissue cultures used for propagation of viruses, and observed no deleterious effects of Fungizone on viral multiplication in the infected cells or of cell

multiplication of the uninfected cells. We have studied the effect of Fungizone on growth of a number of established cell lines and of chick fibroblasts in monolayer culture, and have confirmed that Fungizone when used in certain fungistatic concentrations, does not noticeably affect cell multiplication. We have also studied the stability of Fungizone in a number of tissue culture media and determined rate of inactivation at 37°C in these menstra.

Methods. Four media were used in these experiments including Eagle's medium(6) with 10% (v/v) calf serum, Ziegler's modification (7) of Eagle's medium containing 10% (v/v) calf serum, Waymouth's chemically defined medium MB 752/1(8) supplemented with 10% (v/v) calf serum and 0.03% carboxymethylcellulose and Waymouth's medium supplemented with 0.5% carboxymethylcellulose. Among the cell lines used in these studies were Earle's L₉₂₉ strain of mouse fibroblasts, the HeLa (Gey) cell line, a cell line derived from bovine pituitaries, and 2 cell lines derived from samples of peritoneal fluid from mice infected with Ehrlich ascites tumors. Chick fibroblasts were prepared by

* Fungizone is the registered trade mark of E. R. Squibb and Sons for the amphotericin B sodium deoxycholate complex.

trypsinization of minced tissue and grown in Eagle's medium in monolayers.

Milk dilution bottles (40cm² surface) and Falcon plastic flasks (25 cm² surface) were used for the monolayer cultures, while suspension cultures were grown in 1 × 6 inch screw capped tubes placed on the Rollordrum apparatus as described earlier (9). Cell counts were determined with a Coulter Counter and ID₅₀ values determined graphically from dose response curves. Inoculum for the suspension cultures was adjusted to 100,000 cells per ml and after 3 to 5 days' incubation the populations in the control tubes had increased to between 450,000 and 750,000 cells per ml depending on the cell line. Smaller inocula were used for the cultures grown in monolayers, and microscopic observations were made at 2 day intervals to determine the effect of Fungizone on cell morphology and multiplication.

Fungizone concentrations of supplemented media were determined by a bioassay using *Saccharomyces mellis* as test organism (10).

Results. A study of the stability of Fungizone in tissue culture media incubated up to 7 days at 37°C is summarized in Table I. The presence of serum in the media apparently stabilized the antifungal agent, and the presence of growing cells had no effect on the stability of Fungizone in these systems. As most cultures of yeasts and fungi are com-

pletely inhibited by 1 µg of Fungizone per ml, there still would be sufficient antifungal activity left after 7 days' incubation of media initially containing 5 µg Fungizone per ml, and a great excess when the initial concentration was 50 µg per ml. No loss of antifungal potency was found when these media supplemented with 5 or 50 µg per ml of Fungizone were stored at 4°C for 7 days, and no precipitation of the antifungal agent from the medium was observed.

The effectiveness of Fungizone in inhibiting growth of yeasts and fungi added to Eagle's tissue culture medium was also examined. Among the cultures used in these experiments were *Aspergillus niger*, *Penicillium notatum*, unidentified *Penicillium* and yeast cultures isolated from contaminated tissue culture, *Rhizopus arrhizus*, *Saccharomyces cerevisiae* and *Candida albicans*. A concentration of 0.5 µg of Fungizone per ml was effective in inhibiting the growth of the fungi and yeasts when initial inocula were 10,000 and 100,000 mold spores per ml, and 100,000 and 1 million yeast cells per ml. Cultures were incubated at 37°C for 4 to 7 days. These results confirm and extend the observations reported by Hemphill *et al.* (5).

Observations on the sensitivity of the cell lines to Fungizone are summarized in Table II. Most of the cultures when grown in ser-

TABLE I. Stability of Fungizone in Tissue Culture Media Incubated at 37°C.

Medium	% of antifungal activity* remaining after incubation for stated period at 37°C							
	Initial conc. 5 µg/ml				Initial conc. 50 µg/ml			
	Medium without cells		Medium with cells†		Medium without cells		Medium with cells†	
	4 days	7 days	4 days	7 days	4 days	7 days	4 days	7 days
Eagle's medium containing 10% (v/v) calf serum	39	20	57	42	78	67	80	76
Ziegler's modification of Eagle's medium with 10% (v/v) calf serum	56	40	40	32	74	67	78	71
Waymouth's medium MB 752/1 supplemented with 10% (v/v) calf serum	54	34	65	38	58	58	33	25
Waymouth's medium MB 752/1 supplemented with 0.5% carboxymethyl-cellulose	13	11	18	18	73	54	66	57

* As determined by bioassay using *Saccharomyces mellis* as test organism.

† Medium inoculated with Earle's L₉₂₉ culture of mouse fibroblasts.

TABLE II. Effect of Fungizone® on Multiplication of Cells in Tissue Culture.

Cell line	Method of growth	Medium	ID ₅₀ , µg/ml	Other observations
L ₉₂₉ (mouse fibroblast)	Suspension	A	25	
	"	B	2.5	
	"	D	30	
	Monolayer	A		No inhibition at 25 µg/ml
HeLa	Monolayer	A		<i>Idem</i>
Bovine pituitary	Suspension	A	25	
Ehrlich ascites (R)	"	A	35	Slight inhibition at 5 µg/ml
" " (S)	"	A	35	<i>Idem</i>
Chick fibroblast	Monolayer	C		No inhibition at 25 µg/ml

Composition of media: A—Waymouth's MB 752/1 supplemented with 10% (v/v) calf serum.
 B—Waymouth's MB 752/1 supplemented with 0.3% carboxymethylcellulose.
 C—Eagle's medium containing 10% (v/v) calf serum.
 D—Ziegler's modification of Eagle's medium with 10% (v/v) calf serum.

um-containing media were not inhibited by addition to the media of 2.5 µg to 5 µg of Fungizone per ml. Some of the cytotoxicity of Fungizone is perhaps due to the deoxycholic acid component of the preparation as growth of most of the cell lines tested was found to be at least slightly inhibited by 25 µg per ml (11). Amphotericin B (dissolved in dimethylformamide) caused 50% inhibition of growth of L cells in suspension when added to the medium at a concentration of 50 µg per ml. This inhibition was associated with the amphotericin B as addition of an equivalent amount of dimethylformamide (0.004 ml per ml of medium) did not affect cell growth.

In experiments studying the effectiveness of Fungizone in eliminating growth of yeasts from tissue culture, approximately 1,000 *S. cerevisiae* cells were added to a 25 ml suspension culture of L cells contained in 1 × 6 inch screw-capped test tubes. After 16 hours incubation on the Rollordrum apparatus the yeast cell count had reached about 1,000,000 cells per ml. Fungizone was then added to several of the tubes to give concentrations ranging from 2.5 to 25 µg per ml and the tubes replaced on the Rollordrum apparatus. After 24 hours incubation the yeast count in all the tubes had dropped noticeably. The tubes were centrifuged, the collected cells resuspended in media containing 2.5 µg of Fungizone per ml, and the tubes replaced on the Rollordrum apparatus. After 48 hours incu-

bation the L cell count had doubled and no evidence was seen of viable yeast on microscopic inspection. The L cells were again collected by centrifugation, and resuspended in media without Fungizone or other antifungal agents. No evidence of yeast cell growth was found after 3 days' incubation, and the L cells appeared "normal" when examined microscopically. In another study, a penicillium culture was found growing in a suspension culture of a cell line taken from bovine pituitary tissue. Addition of Fungizone to give a concentration of 2.5 µg/ml resulted in inhibition of growth of the penicillium, and after 2 subcultures (at 3 day intervals) the cell line was found to be free of the contaminant. Fungizone at 2 µg/ml was also effective in inhibiting growth of another penicillium contaminant found in a monolayer culture of a cell line derived from Ehrlich ascites cells. Since 0.5 µg of Fungizone per ml of medium is sufficient to control fungal and yeast contamination, we conclude that the use of 2 µg to 5 µg per ml of medium may be used to eliminate fungal and yeast contaminants from tissue cultures without significantly damaging the tissue cultures. Continued use of media supplemented with serum and 10 µg of Fungizone per ml of medium for growth of Earle's L₉₂₉ cell line in suspension cultures did not result in any change in cell multiplication rates during the 3 week observation period.

Summary. In experiments studying the effect of Fungizone (the sodium deoxycholate

complex of amphotericin B) on growth of established cell lines and chick fibroblasts in monolayer and suspension culture systems, the fungistatic concentration of 2.5 $\mu\text{g/ml}$ was found to have little or no effect on the multiplication of the tissue cultures grown in serum-containing media. This concentration was tolerated by all cultures. Inactivation of Fungizone in tissue culture media was also determined.

1. McLimans, W. F., Bonissol, C., Davis, E. V., Rake, G., in *Antibiotics Annual*, 1955-1956, 690, New York, Medical Encyclopedia, Inc.
2. Bartner, E., Zinnes, H., Moe, R., *ibid.*, 1956-1957, 866.
3. Dutcher, J. D., Gold, W., Pagano, J. F., Van-

deputte, J., U. S. Patent 2,906,611 (1959).

4. Gold, W., McKee, G. M., Pagano, J. F., Donovan, R., in *Antibiotics Annual* 1955-1956, 579, New York, Medical Encyclopedia, Inc.
5. Hemphill, J. J., Herman, Y. F., Young, V. M., *ibid.*, 1957-1958, 961.
6. Eagle, H., *Science*, 1955, v122, 501.
7. Ziegler, D. W., Davis, E. V., Thomas, W. J., McLimans, W. F., *Appl. Microbiol.*, 1958, v6, 305.
8. Waymouth, C., *J. Nat. Cancer Inst.*, 1959, v22, 1003.
9. Perlman, D., Giuffre, N. A., Jackson, P. W., Giardinello, F., *PROC. SOC. EXP. BIOL. AND MED.*, 1959, v102, 290.
10. Gerke, J. R., Madigan, M. E., *Antibiot. and Chemother.*, 1961, in press.
11. Perlman, D., Giuffre, N. A., unpublished.

Received January 20, 1961. P.S.E.B.M., 1961, v106.

Carbohydrate Content of Euglobulins of Normal and Rheumatoid Sera. (26510)

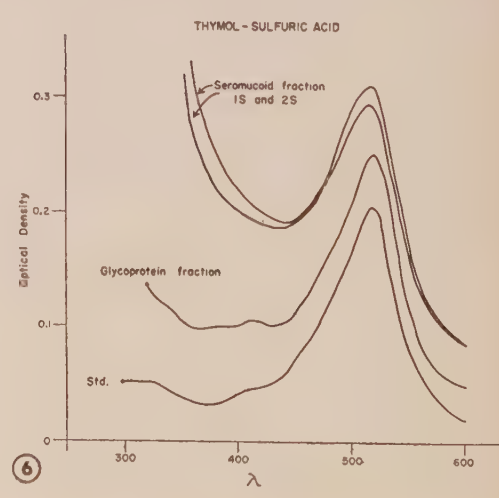
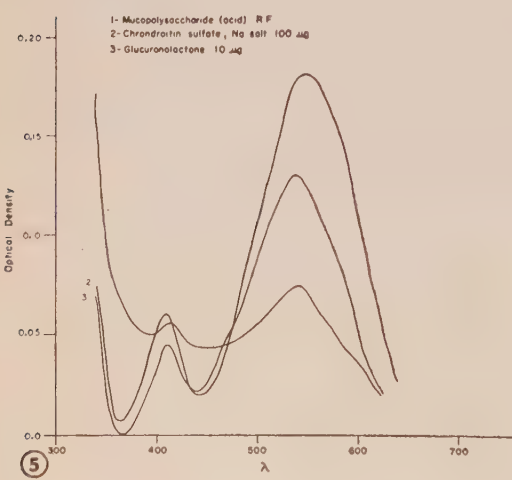
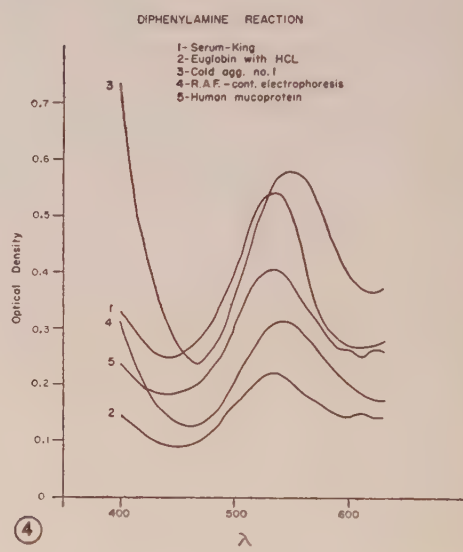
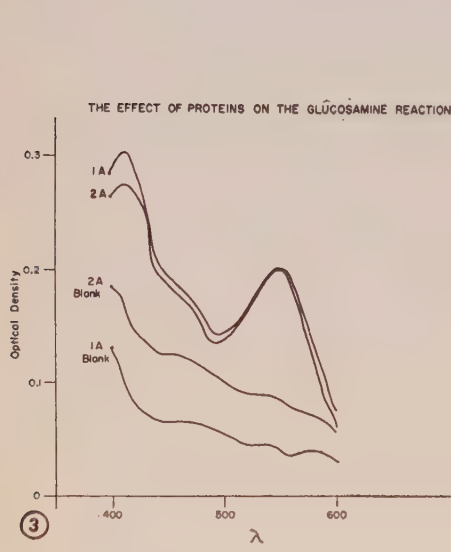
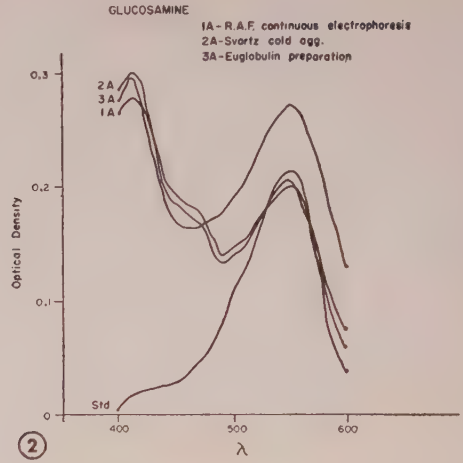
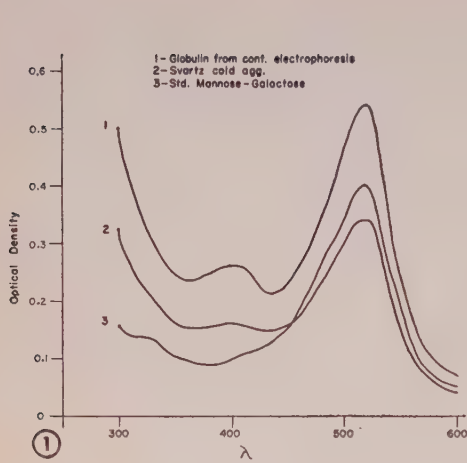
WILLARD R. STARNES (Introduced by Emmett B. Carmichael)

Metabolic Research Laboratories, Veterans Administration Hospital, Birmingham, Ala.

The term "rheumatoid factor" has been applied to the protein component of serum which is responsible for a group of agglutination and precipitation reactions, some of which are very important in diagnosis of rheumatoid arthritis. Up to the present, analytical studies have indicated that the rheumatoid factor is a typical 19-S gamma globulin with chemical, physical and immunological properties closely akin to 19-S antibodies(1). The present studies were undertaken to isolate euglobulins by different methods and study the carbohydrate composition. Franklin, Muller-Eberhard and Kunkel isolated and characterized, by various methods, a refined and more purified "rheumatoid factor" preparation upon which they determined the various carbohydrate fractions(1). Others have also achieved considerable purification by other technics(2,3).

Materials and methods. Known normal pooled sera and pooled sera with a positive latex agglutination were submitted to 3 different methods for isolation and purification of the euglobulin fractions. (1) *Precipitation*

with 0.0027 N hydrochloric acid: Test sera were inactivated by heating at 56°C for one-half hour. In a 100 ml cellulose nitrate tube, one volume of clear serum was diluted by the slow addition (with continuous rotation of the tube) of 9 volumes of 0.0027 N hydrochloric acid from a 50 ml burette. A pH within the range of 6.1 to 6.4 was thereby attained. The tubes were immediately placed in a refrigerated centrifuge at 4 to 8°C for 30 to 60 minutes. The euglobulin precipitate then was centrifuged in the cold at 3,000 rpm for 10 minutes. The clear supernate was discarded by decanting from the firmly packed pellet, and last traces of the supernate were removed either by draining the tube inverted over filter paper or by wiping the inner wall thoroughly with a gauze sponge. The precipitate was not washed. The concentrated euglobulin was then dissolved in an added 0.5 ml of 0.9% NaCl solution, (2) Svartz cold agglutination, (3) and isolation and purification by continuous electrophoresis in veronal buffer 0.05 M pH 8.6. The euglobulin was subjected to 3 separations on



the Spinco continuous electrophoretic apparatus. Following euglobulin isolation all samples were tested with latex(4). Each euglobulin was then subjected to paper electrophoresis for protein and carbohydrate content on a Spinco Model R electrophoretic apparatus using veronal buffer 0.05 M, pH 8.6. All the above samples were gamma globulin in nature with the exception of one prepared with hydrochloric acid which contained 1.5% beta globulin.

The following tests were made for carbohydrate:

(1) Total protein bound hexose (T.P.B.H.). The thymol-sulfuric acid reaction(5). Isolation of acid mucopolysaccharide by the method of Bollet(6). (2) Hexosamine by a modification of the Elson-Morgan method(7). (3) Sialic acid by the method of Pigman, *et al.*(8). (4) Acid mucopolysaccharides by the carbazole method of Dische(9).

Results and discussion. In Fig. 1, 2 of the euglobulin preparations are compared with a mannosegalactose standard of 40 μ g (20 μ g of each). The 2 globulins exhibit a typical peak in the 420 $m\mu$ range which is due to presence of protein. Carbohydrate moiety shows maximum absorption at 520 $m\mu$. This is in agreement with Shetlar and Masters(5) who found the protein curve absorbs more in the 420-440 $m\mu$ range of the curve. Absorp-

tion curves obtained when sulfuric acid reacts with protein (in absence of thymol) has some absorption at 420 $m\mu$, most of the effect is apparently due to the reaction of sulfuric acid with proteins and does not involve the reaction with thymol.

In Fig. 2 the 3 different preparations were compared with a standard of glucosamine hydrochloride, maximum absorption being the same as glucosamine at 550 $m\mu$. The increase in optical density from 400 to 450 $m\mu$ was due to the hydrolytic products of protein. This could be reduced greatly if hydrolyzed proteins were used as blanks. No attempt was made to purify further the glucosamine hydrolyzates because the use of the blank corrected for the increase in optical density (Fig. 3).

In Fig. 4 the euglobulin fractions were examined for their sialic acid content, utilizing the diphenylamine reaction. Specimen No. 5 was purified human glycoprotein preparation which contained 12% sialic acid. Also included was a serum to serve as a control for the reaction which was found to contain 114 mg% of sialic acid. All of the euglobulins exhibited a peak at 530 $m\mu$ as did the serum and the purified human mucoprotein fraction with the exception of No. 3 which was a cold agglutination prepared by the method of Svartz(3). This was repeatedly checked and

FIG. 1. Absorption spectrum of total protein-bound hexoses in thymol-sulfuric acid. (1) Euglobulin from continuous electrophoresis. Total protein bound hexoses, 49.8 μ g. (2) Euglobulin prepared by Svartz cold agglutination method. Total protein bound hexoses, 34.3 μ g. (3) Mannose-galactose standard 40 μ g (20 μ g each of mannose and galactose).

FIG. 2. Glucosamine absorption spectrum of euglobulin preparations. (1A) Euglobulin isolated from latex positive serum by continuous electrophoresis. Glucosamine, 53 μ g. (2A) Euglobulin isolated from latex positive serum by Svartz cold agglutination method. Glucosamine, 57 μ g. (3A) Euglobulin from latex negative serum. Glucosamine, 74 μ g. (4) Standard (Std.). Glucosamine, 60 μ g.

FIG. 3. The effect of proteins on the glucosamine reaction. 1A, 2A. Euglobulins isolated by continuous electrophoresis and Svartz cold agglutination method respectively—containing 53 μ g and 57 μ g. Read against Reagent Blank. 1A, 2A Blank. Hydrolytic effect of acid on proteins using distilled water as a blank.

FIG. 4. Diphenylamine reaction for sialic acid. (1) Untreated serum control, .57 mg sialic acid. (2) Euglobulin isolated with HCl, .23 mg sialic acid. (3) Svartz cold agglutination euglobulin, .56 mg sialic acid. (4) Euglobulin isolated by continuous electrophoresis, .29 mg sialic acid. (5) Mucoprotein, purified (human), .52 mg sialic acid.

FIG. 5. Carbazole reaction for uronic acids. (1) Mucopolysaccharide (acid) R.F. isolated from latex positive euglobulin, 5.1 μ g. (2) Chondroitin sulfate, sodium salt, 100 μ g. (3) Glucuronolactone, 10 μ g.

FIG. 6. Absorption spectrum of purified glycoprotein and seromucoid fractions utilizing the thymol-sulfuric acid reaction. (1S) Seromucoid fraction isolated with phosphotungstic acid from a latex positive euglobulin, 60.5 μ g. (2S) Seromucoid fraction isolated with protamine sulfate from a latex positive euglobulin, 57.6 μ g. Glycoprotein fraction isolated with perchloric acid from a latex positive euglobulin. Std. = Mannose-galactose standard, 40 μ g.

TABLE I. Protein and Carbohydrate Composition of Euglobulins.

Euglobulin preparations	Total protein, g %	Total protein bound hexoses, mg %	Glucosamine, mg %	Sialic acid, mg %
Continuous electrophoresis	2.7	84.8	78.0	29.1
.0027 N HCl, pH 6.1-6.4	1.2	68.4	33.5	23.3
Cold agglutination (Svartz)	2.9	86.1	76.5	55.9
Normal euglobulin (10 donors)	1.2	33.6	18.3	16.5
Whole serum (10 donors)	7.9	182.0	—	114.0

found to show maximum absorption at 550 $m\mu$.

When the carbazole reaction was applied to a product isolated by classical procedures for acid mucopolysaccharide-like substance (Fig. 5) it was found to have maximum absorption at 540 $m\mu$ the same as glucuronolactone and chondroitin sulfate. The cold agglutination euglobulin used for isolation of the acid mucopolysaccharide contained 144.0 mg of total protein with a total protein bound hexose of 5.6 mg. This yielded 10.2 μ g of acid mucopolysaccharide calculated as chondroitin sulfate. Protamine gave no color with the carbazole reaction, confirming the results observed by Bollet(6). Badin and Schubert added chondroitin sulfate to plasma and found it in the euglobulin fraction(10).

Badin and Schubert(10) isolated euglobulin with a phosphate-citrate buffer, then precipitated the uronic acid with lysozyme. If the above is chondroitin sulfate it is not in agreement with Badin and Schubert as they obtained higher values in rheumatoid euglobulins possibly due to the method of isolation of the acid mucopolysaccharide-like substance.

No attempt was made to identify the acidic component because of the limited amount present. Using the procedure of Bollet(6) and the carbazole method for uronic acids it appeared to be a polysaccharide containing uronic acid which exhibits the characteristics of chondroitin sulfate. In the separation of the uronic acid-containing mucopolysaccharides, the positive latex reaction was lost following reprecipitation of the euglobulin with 1.8 M perchloric acid probably due to denaturation. In checking each component from the fractionation of the acid mucopolysaccharide a loss of 14.1 mg of protein and 1.6 mg total protein bound hexose was encountered.

The supernatant from the citric-phosphate buffer was dialyzed against tap water for 24 hours and 0.5 ml of 18% phosphotungstic acid was added to obtain a seromucoid fraction. The precipitate was washed with cold ethanol, then dissolved in 5.0 ml containing 1.0 ml of 1% sodium carbonate and 4.0 ml phosphate-saline buffer pH 8.2. This gave a protein high in glycoprotein material with a hexose:protein ratio at 1:5 whereas the original euglobulin was 1:25. The seromucoid fraction gave a positive thymol-sulfuric reaction similar to those observed with the original euglobulin total protein bound hexose.

In Fig. 6, "1 S" represents the seromucoid fraction treated with phosphotungstic acid and "2 S" is the same except prior treatment with protamine sulfate for precipitation of the uronic acid-containing mucopolysaccharide. The glycoprotein curve represents the precipitate following initial treatment with 0.14 M NaCl in 0.02 M NaOH and 1.8 M perchloric acid. The standard is made up with equal quantities of mannose and galactose.

Table I gives results of several analytical methods used on the various euglobulin fractions that we have isolated. The percentage of total protein bound hexoses, glucosamine and the sialic acid present in each of the euglobulin fractions is given, with mg% of each. The fractions isolated were gamma globulins by paper electrophoretic means. When these values were compared with those of normal euglobulin a marked elevation was observed.

Summary. 1. Euglobulins were prepared by 3 different methods from "latex positive" pooled serum. 2. Total protein bound hexoses, glucosamine, and sialic acid were measured on each euglobulin preparation. All were some-

what similar with the exception of the euglobulin prepared with hydrochloric acid, which had a higher per cent of hexose to protein. All showed marked increase in content when compared with euglobulins isolated from a pooled "latex negative" normal serum. 3. Paper electrophoretic examination reveals proteins that migrate as gamma globulins and are highly PAS positive. 4. A uronic acid-containing mucopolysaccharide was isolated from the cold agglutinating euglobulin.

1. Franklin, E. C., Holman, H. R., Muller-Eberhard, H. J., Kunkel, H. G., *J. Exp. Med.*, 1957, v105, 425.

2. Kunkel, H. G., Franklin, E. C., Muller-Eberhard, H. J., *J. Clin. Invest.* 1959, v38, 424.

3. Svartz, N., *Ann. Rheumat. Dis.*, 1957, v16, 441.

4. Singer, J. M., Plotz, C. M., *Am. J. Med.*, 1956, v21, 88.

5. Shetlar, M. R., Masters, Y. F., *Anal. Chem.*, 1957, v29, 402.

6. Bollet, A. J., Seraydarian, M. W., Simpson, W. F., *J. Clin. Invest.*, 1957, v36, 1328.

7. Winzler, R. J., *Methods of Biochemical Analysis*, 1955, v2, 279, Interscience Publishers, N. Y.

8. Pigman, W., Hawkins, W. L., Blair, M. G., Holley, H. L., *Arthritis and Rheumatism*, 1958, v1, 151.

9. Dische, Z., *J. Biol. Chem.*, 1947, v167, 189.

10. Badin, J., Schubert, M. J., *J. Clin. Invest.*, 1955, v34, 1317.

Received January 30, 1961. P.S.E.B.M., 1961, v106.

Estrogens and Progesterone in the Sea Urchin (*Strongylocentrotus franciscanus*) and Pecten (*Pecten hericius*).^{*} (26511)

CHARLES R. BOTTICELLI, FREDERICK L. HISAW, JR.,[†] AND HERBERT H. WOTIZ[‡]
(Introduced by Frederick L. Hisaw)

Biological Laboratories, Harvard University, Cambridge, Mass., Department of Biochemistry, Boston University School of Medicine, and University of Washington, Friday Harbor Marine Station

Estradiol-17 β , estrone, and progesterone have been obtained, in vertebrates other than mammals, from the ovaries of dogfish(1), while estradiol, estrone, and estriol have been found in the ovaries of laying hens(2) and lungfish(3). Extracts of gonads from many invertebrates have produced estrogenic effects in mammals(4,5), but the chemical identity of the active substances is mostly unknown. Only recently estradiol and progesterone have been identified in the ovaries of the starfish(6). This report presents the results of an effort to isolate and identify possible estrogenic steroids and progesterone from ovarian tissue of 2 other representative invertebrates: the sea urchin, *Strongylocentrotus franciscanus*, and the mollusk, *Pecten hericius*.

^{*} Aided by grants from Nat. Science Foundation and U.S.P.H.S.

[†] Dept. of Zoology, Oregon State College, Corvallis.

[‡] Senior Research Fellow, U.S.P.H.S.

Methods and results. The ripe ovaries (3-5 kg) of each species were collected and stored in approximately twice their volume of acetone. The acetone extract was evaporated to an oily residue, taken up by 70% ethanol and extracted 3 times with petroleum ether. Next, the ethanolic fraction was diluted with water to contain 35% of alcohol and washed with petroleum ether 3 times. These 35% ethanolic fractions were tested for estrogenic action by the Astwood method, as previously described(7,8), and all gave positive results, although it was evident that only low concentrations of hormone were present. The ethanolic fraction was then dried *in vacuo* and the residue partitioned countercurrently between 70% methanol and a 50-50 mixture of chloroform and carbon tetrachloride with 29 transfers. In brief, those tubes corresponding to authentic estradiol, estrone, and estriol were pooled and bioassayed. The results (Table I) indicated estrogenic activity only in material obtained

TABLE I. Results of Bioassay from Countercurrent Separation.

Fractions	Sea urchin		Pecten	
	Uterine wet wt, mg	% increase over controls	Uterine wet wt, mg	% increase over controls
Controls*	22.8		22.8	
0-2	22.3	0	21.6	0
3-9	21.9	0	24.8	9.2
10-19	24.9	9.2	25.7	13.0
20-26	22.2	0	20.3	0
27-29	21.7	0	21.4	0

* Avg uterine wet wt for 20 untreated animals.

from tubes 10-19 for the sea urchin, and tubes 3-9 (estrone) and 10-19 (estradiol) for the pecten. The estrone and estradiol fractions from each species were divided into 3 aliquot portions, and each portion was subjected to the following 3 paper chromatographic systems, at room temperature: (1) benzene-formamide (1:1) for 22-24 hours; (2) toluene-propylene glycol (1:1) for 6 hours; and (3) ligroin-propylene glycol (1:1) for 64 hours. In each of these systems the unknown was applied to one of 4 strips, estrone to another, estradiol to a third, and a mixture of estrone and estradiol to the fourth. The 4 strips in each system were run simultaneously. The 3 strips treated with the authentic estrogens were stained with a ferric chloride-ferricyanide stain. The areas on the experimental strips corresponding to the standards were cut and eluted 5 times with absolute ethanol. Similar elutions also were made of the remaining paper from the experimental strips. An aliquot portion of each eluate was bioassayed. The sea urchin, like the previously tested starfish(6), upon bioassay indicated

the presence of estradiol-17 β , while the test fractions for the pecten corresponding to both estradiol and estrone gave positive results (Table III). Assuming that estradiol can be converted to estrone in the echinoderms, it seems quite probable that estrone also might have been present, but in a quantity insufficient for detection by our methods. It has been estimated for purposes of comparison that the ripe ovaries of starfish and sea urchin contain a minimum of 0.04 to 0.1 μ g of estradiol-17 β per kilo of fresh tissue, while in the pecten there is a minimum of 10.0 μ g.

The brown oily residue obtained on evaporation of the original petroleum ether fractions was used for isolation of progesterone by procedures previously described(1). In brief, the experimental material was subjected to column chromatography. Those fractions that corresponded to authentic progesterone on a control column gave positive results by the Hooker-Forbes method. Other progestational substances were also present, one of which was identified as probably being Δ^4 -3-keto-pregnen-20 β -ol. The evidence for progesterone, though not as convincing as that for estradiol and estrone, is nevertheless significant. The amount of active material obtained was too small for a more thorough characterization, and absolute chemical identification will require further study.

Conclusions. It has become well established that estradiol-17 β , estrone, and progesterone are present in the ovaries of vertebrates from mammals to fishes, and the present report extends these observations to include certain of the invertebrates. Therefore,

TABLE II. Sea Urchin—Bioassay of Eluted Paper Strips from 3 Different Chromatographic Systems.

Chromatographic systems	CC fractions 10-19 chromatography*			
	Estrone		Estradiol	
	Uterine wet wt, mg	% increase over control	Uterine wet wt, mg	% increase over control
Benzene-formamide	21.7	0	27.2	19
Toluene-propylene glycol	22.2	0	25.3	13
Ligroin-propylene glycol	23.6	0	41.5	82

* Areas of test strips corresponding to authentic estrone and estradiol-17 β uterine weights are avg for 2 animals in each experimental test. Avg uterine wet wt for 20 untreated control animals was 22.8 ± 0.3 mg. Tests for tubes 3-9 gave uniform negative results, so were not recorded.

TABLE III. Pecten—Bioassay of Eluted Paper Strips from 3 Different Chromatographic Systems.

Chromato- graphic systems	CC fractions 3-9 chromatography*				CC fractions 10-19 chromatography*			
	Estrone		Estradiol		Estrone		Estradiol	
	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase
Benzene-forma- mide	27.5	20.6	21.3	0	25.8	13.1	35.2	54.8
Toluene-propylene glycol	29.2	28.0	21.5	0	22.2	0	43.8	92.1
Ligroin-propylene glycol	34.1	49.5	22.3	0	21.6	0	45.3	98.6

* Areas of test strips corresponding to authentic estrone and estradiol-17 β . Uterine weights are avg for 2 animals in each experimental test. Avg uterine wet wt for 20 untreated control animals was 22.8 \pm 0.3 mg.

it seems a distinct possibility that they may be of common occurrence in the animal kingdom. It is known, of course, that these compounds have important endocrine functions in the vertebrates, but whether this is also true of the invertebrates is at present unknown. However, there is now considerable evidence indicating that these steroids may take an important part in growth and development of the vertebrate Graafian follicle(9). It seems possible that this may represent the original and hence most primitive function of these substances, and that their action as hormones affecting structures beyond the confines of the ovary may represent later specializations.

Summary. The ovaries of the sea urchin, *Strongylocentrotus franciscanus*, and the pecten, *Pecten hericius*, were extracted for steroids. Estrogenic activity equivalent to 10 μ g or less per kilo of wet tissue was found in each species by bioassay. The most active estrogenic substance was identified as estradiol-17 β . Separations were made by counter-current distribution (29 transfers: upper phase—70% methanol; lower phase—50% chloroformic, 50% carbon tetrachloride), paper chromatography (3 systems: toluene-propylene glycol; ligroin-propylene glycol; ben-

zene-formamide), and bioassay (Astwood method). Progesterone was also identified tentatively by column chromatography and the Hooker-Forbes assay. Other progestational substances were present, one of which may be Δ^4 -3-keto-pregnen-20 β -ol. The occurrence of these steroids in 2 distinctly different phyla indicates a wide distribution and may be of some significance in the evolution of sex hormones.

1. Wotiz, H. H., Botticelli, C. R., Hisaw, F. L., Jr., Olsen, A. G., *Proc. Nat. Acad. Sci.*, 1960, v46, 580.
2. Layne, D. S., Common, R. H., Maw, W. A., Fraps, R. M., *Nature*, 1958, v181, 351.
3. Dean, F. D., Jones, I. C., *J. Endocrin.*, 1959, v18, 366.
4. Hanstrom, B., *Hormones in Invertebrates*, Oxford Univ. Press, 1939.
5. Hagerman, D. D., Wellington, F. M., Villee, C. A., *Biol. Bull.*, 1957, v112, 180.
6. Botticelli, C. R., Hisaw, F. L., Jr., Wotiz, H. H., *Proc. Soc. Exp. Biol. and Med.*, 1960, v103, 875.
7. Astwood, E. B., *Endocrinol.*, 1938, v23, 25.
8. Hisaw, F. L., Jr., *ibid.*, 1959, v64, 276.
9. *Marshall's Physiology of Reproduction*, A. S. Parkes, Ed., Longmans, Green and Co., London, 1956, vI, Pt. I, Chap. 5.

Received February 20, 1961. P.S.E.B.M., 1961, v106.

Induction of Leukemia in Rats with Mouse Leukemia (Passage A) Virus.* (26512)

LUDWIK GROSS

(With technical assistance of Yolande Dreyfuss and Lorraine A. Moore)

Cancer Research Unit, Vet. Administration Hospital, Bronx, N. Y. City

A mouse leukemia virus (passage A) originally isolated from spontaneous Ak leukemia and then passed by serial cell-free passage through newborn C3H mice(1) was employed in this study. When inoculated into less than 10-day-old C3H/Bi mice, this virus, now in its 27th passage, consistently induces lymphatic leukemia in over 95% of the inoculated animals after an average latency of less than 3 months.

An attempt was made to determine whether this mouse leukemia virus could also induce leukemia in rats.

Materials and methods. Animals. From a nucleus of randomly bred Sprague-Dawley rats received in June 1960 through the courtesy of Dr. J. B. Moloney from the Animal Production Unit, Nat. Inst. of Health, Bethesda, Md., a small colony of rats has been raised in our laboratory by brother-to-sister mating.

Leukemic extracts of 20% concentration were prepared in the usual manner(1) from thymic and mesenteric lymphoid tumors, as well as spleens and livers pooled from several C3H/Bi mice with passage-A-virus-induced leukemia.

Following centrifugation, at 0°C, at 3,000 rpm for 15 minutes, then at 9,500 rpm for 5 minutes, the final supernate was passed through Selas, porosity 02, filter candles, and immediately used for inoculation of newborn, less than 1 day old, Sprague-Dawley rats. All inoculations were intraperitoneal (0.5 ml each). The animals were weaned when about 3 weeks old, separated by sexes, and kept for observation.

Experimental. In the first experiment, a newborn litter consisting of 6 females and 5 males was inoculated with the leukemic filtrate. Eight of the 11 rats (73%) developed

leukemia at 3 to 4 months of age; the remaining 3 rats are still in good health at 5 months of age. Among the leukemic rats, 3 had very large thymic lymphosarcomas filling out almost the entire chest cavity; 3 other rats had very large thymic lymphosarcomas, but also very large spleens, large livers, and moderately large mesenteric lymphosarcomas; the remaining 2 rats had a generalized stem-cell leukemia (Fig. 1). No enlargement of inguinal or axillary lymph nodes was noticed in any of these animals. Only 2 rats had a high peripheral (tail) white blood count; the highest count was 403,000 WBC per cu mm with 95% stem cells (Fig. 2). In other rats, the peripheral blood changes were moderate, with slight, if any, elevation of the white cell counts and only occasional presence of blast cells; all leukemic rats, however, had anemia.

In a second experiment, a litter consisting of 7 newborn rats was inoculated with the leukemic filtrate; 4 rats (57%), thus far, have developed leukemia at 4 to 4½ months of age. One had a generalized stem-cell leukemia (188,000 WBC per cu mm), and one a generalized lymphatic leukemia; 2 had very large thymic lymphosarcomas with no other pathology. The remaining 3 rats now 5 months old, are still in good health.

In a third experiment, now in progress, 3 newborn litters, consisting of a total of 21 rats, were inoculated with the leukemic filtrate; 12 rats have thus far (57%) developed leukemia at 2½ to 3 months of age; the remaining 9 are still in good health, but are only 3 months old now. Among the leukemias observed, 4 were thymic lymphosarcomas, 7, were generalized lymphatic leukemias and one was a stem-cell leukemia.

Discussion. Experiments reported here suggest that newborn rats of the Sprague-Dawley strain are susceptible to the leukemogenic action of the mouse-leukemia-derived

* Aided in part by grants from Damon Runyon Memorial Fund and Am. Cancer Soc.



FIG. 1. Rat of Sprague-Dawley strain inoculated (.5 ml i.p.) when less than 1 day old with mouse leukemia (passage A) filtrate. As a result, generalized leukemia developed after a latency of 4 mo. Note large mediastinal and mesenteric tumors, very large spleen, and large liver. Blood examination showed stem-cell leukemia (see Fig. 2). Bone marrow was also leukemic.

passage A virus. The incidence of leukemia thus far induced in rats varied from 57 to 73%; however, among the inoculated animals, those that remained free from leukemia are still young, and some may develop leukemia later. It is our impression that final incidence will exceed 80%, and will thus be only slightly lower than that observed following inoculation of the same virus into suckling mice of susceptible strains. Rats of the Sprague-Dawley strain are not homogeneous, and susceptibility may vary from one litter to another. The incidence of spontaneous leukemia in rats of this stock is very low, and probably does not exceed 2% in old animals, but is difficult to assess since no exact data

are available. We have not yet seen a spontaneous leukemia among our untreated animals of this strain.

Whereas it is relatively easy to recognize leukemia in the live mouse, the difficulties are more pronounced in the rat. Labored breathing may be indicative of a thymic lymphosarcoma, but is diagnostic only in more advanced phases of the disease. Enlarged spleen is another sign of certain forms of generalized leukemia in the rat, but is not always present; furthermore, it may not be easy to palpate the abdomen of adult rats and detect a moderate spleen enlargement. Blood counts are reliable as a diagnostic aid, but only in cases in which leukemia involves the peripheral blood; quite frequently, however, the disease may be aleukemic. Thus the investigator must frequently base his diagnostic considerations in live rats not only on peripheral blood examination, but also on the general appearance and behavior of the animals, their relative strength, weight, and manner of breathing. Curiously, the inguinal and axillary lymph nodes were not prominently enlarged in most leukemic rats thus far observed in our studies, thus adding to diagnostic difficulties; in mice, the often, although not always, prominently enlarged peripheral lymph nodes in either spontaneous, or filtrate-induced, leukemia facilitate diagnosis in the live

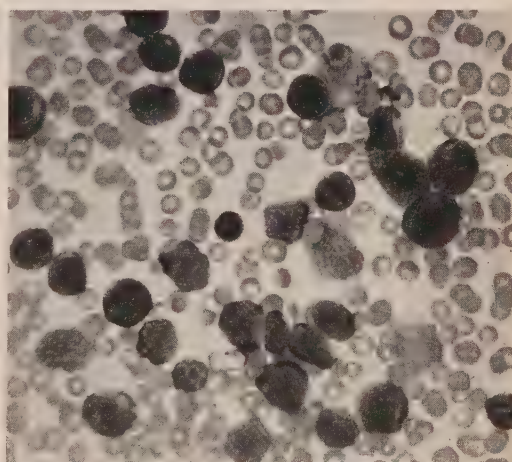


FIG. 2. Blood smear from tail of a rat in which leukemia developed following inoculation of mouse leukemia (passage A) virus (see Fig. 1). WBC 403,000/mm³. 95% stem cells. Magnification 520 × (oil immersion). Wright and Giemsa stain.

animal. No diagnostic difficulties were encountered, however, once the leukemic rats died or were sacrificed, and their internal organs were inspected. The large thymic tumors, frequently enlarged spleens and livers, and mesenteric tumors, could hardly be missed. The blood morphology was diagnostic only in some animals; in others, the disease was aleukemic, with only insignificant changes in blood morphology. Similarly, microscopic sections of internal organs, such as spleen, liver, or kidneys, showed infiltration with leukemic cells only in animals with generalized leukemia. The femoral bone marrow of the leukemic rats was examined in several instances, and was usually found to be leukemic; however, in at least 2 of those leukemic animals in which the disease was limited to very large thymic lymphosarcomas, the bone marrow was found to be non-leukemic. This was consistent with our previous observations on virus-induced mouse leukemia (Gross, unpublished), in which occasionally mice with typical, but early, leukemia had non-leukemic bone marrow. It is possible that the leukemic process may initially develop in the thymus, or in another organ such as spleen, then spread rapidly to other organs of the host, including also the bone marrow. Accordingly, the bone marrow may represent a secondary, and not necessarily a primary, site of the disease.

It is of considerable interest that a mouse leukemia virus could induce leukemia in rats, even though the same virus has been observed to display a specific affinity for particular inbred strains of mice(2). The host specificity of the Ak-leukemia-derived mouse virus was more pronounced in our early studies(3) in which extracts of relatively low potency were employed. Even the more recently employed potent passage virus, however, is still more leukemogenic for certain inbred strains of mice, such as C3H/Bi, or C57 Brown/cd, than for others, such as C3H/An, BALB/c, or A(2). The curious phenomenon was thus observed that a leukemic virus may display specificity for certain strains of mice, or even for certain sublines of inbred strains(3), yet the same virus may be able to cross a species

barrier and induce a similar disease in animals of 2 different species.

Other oncogenic mouse viruses also have been observed to induce leukemia in rats. Graffi and Gimmy(4) observed development of leukemia following inoculation of newborn rats of either Sprague-Dawley, or Wistar strains with a leukemogenic virus isolated from transplanted mouse tumors; the incidence of leukemia, however, induced with this mouse virus in rats was low and averaged only 10%(5). Moloney, employing for inoculation a leukemogenic mouse virus isolated from transplanted mouse sarcoma 37, was able to induce an incidence of leukemia exceeding 70% in rats of the Sprague-Dawley strain(6).

It thus appears that a mouse leukemia virus may induce leukemia also in rats. The tissue-culture-grown parotid tumor (polyoma) virus(7), which is essentially an oncogenic virus originally isolated from leukemic mouse tissues(8), also induces tumors not only in the host of the origin, *i.e.*, in mice, but also in hamsters(9), and rats(10). The oncogenic viruses may be in general much less species-specific than might have been anticipated.

Addendum. As this manuscript goes to press, additional data became available suggesting that the leukemic virus could be readily recovered from rats in which leukemia had been induced with passage A filtrate, and could then be passed from rat to rat. From leukemic organs of one of the rats in which leukemia developed following inoculation of passage A (mouse leukemia) filtrate, a filtered (Selas 02) extract was prepared in the usual manner, and inoculated into 9 newborn Sprague-Dawley rats. After a short latency of only 2 months, 8 out of the 9 inoculated rats (89%) developed leukemia; two rats had very large thymic lymphosarcomas, and the remaining 6 had a generalized lymphatic leukemia.

Summary. 1. A mouse leukemia virus, originally isolated from spontaneous Ak leukemia, then passed serially through newborn mice (passage A), and consistently leukemogenic for suckling mice of susceptible strains, was found to be leukemogenic also for newborn rats. 2. Following intraperitoneal inoculation of passage A filtrates into newborn Sprague-Dawley rats, leukemia developed in

from 57% to 73% of the inoculated animals, after a latency varying from $2\frac{1}{2}$ to $4\frac{1}{2}$ months. 3. Either localized thymic lymphosarcomas, or generalized lymphatic leukemia, involving also spleen, liver, and mesenteric lymph nodes, developed in the inoculated animals. A few rats developed acute stem-cell leukemia.

1. Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 767.

2. ———, *Acta Haemat.*, 1960, v23, 259.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 64.

4. Graffi, A., Gimmy, J., *Naturwissenschaften*, 1957, v44, 518.

5. Graffi, A., *Progr. Exp. Tumor Res.*, Karger Publ., 1960, v1, 112.

6. Moloney, J., *Nat. Cancer Inst. Monograph No.* 4, 1960, 7.

7. Eddy, B. E., Stewart, S. E., Berkeley, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 848.

8. Gross, L., *ibid.*, 1953, v83, 414.

9. Eddy, B. E., Stewart, S. E., Young, R., Mider, G. B., *J. Nat. Cancer Inst.*, 1958, v20, 747.

10. Eddy, B. E., Stewart, S. E., Stanton, M. F., Marcotte, J. M., *ibid.*, 1959, v22, 161.

Received February 21, 1961. P.S.E.B.M., 1961, v106.

Serum Free Cholesterol and Atheroma in Young Cockerels. (26513)

CLYDE T. CALDWELL (Introduced by M. H. Kuizenga)

Nutrition and Metabolic Diseases, Upjohn Co., Kalamazoo, Mich.

Partial conversion of free to combined cholesterol upon incubation of normal human serum has been reported(1). Thirty-one to 50% decrease in free cholesterol occurred when serum from 20 normal patients was incubated(2). Incubation of serum from most patients with active coronary atherosclerosis resulted in less than normal conversion(3). These observations suggested the value of studying the relationship between serum free cholesterol not combined upon incubation and development of atheroma in normal and atherosclerotic young cockerels.

Material and methods. Approximately 4 wk old White Leghorn cockerels were used. Normal birds were fed a commercial chick growing mash *ad lib.** Atherosclerosis was induced by feeding the basal diet containing .5% cholesterol deposited from ethyl ether solution. Atherosclerotic condition was evaluated as % area of the aorta, brachiocephalic, and iliac arteries involved in plaque formation. Pooled blood serum from each group was incubated 30 hr at 37-38°C. Other methods employed have been described(4).

Results and discussion. Extent of artery involvement and serum cholesterol data be-

fore and after incubation were determined in 3 series of groups of birds, representing normal, mild spontaneous, and severe cholesterol-induced atherosclerosis respectively (Tables I, II, III). Total cholesterol values did not change appreciably upon incubation. Last column in each table shows the % original free cholesterol remaining after serum incubation.

Of particular interest is the incubation increase of % uncombined free cholesterol from normal (17 to 45) through mild spontaneous (45 to 56) and severe cholesterol-induced atherosclerosis (57 to 87%). The range, 17 to 45%, for normal birds suggests an increasing tendency toward atherosclerotic development although plaques were not grossly observable (Table I). Corresponding results, 45 to 56%, associated with the first appearance of plaques as mild spontaneous atherosclerosis in birds on normal diet support this suggestion (Table II).

Plaques were always found when more original free cholesterol remained than was combined upon incubation. They were not found when less than 45% remained. Approximately 57% marked the beginning of severe atherosclerosis. This narrow range for mild spontaneous artery involvement represents a

*Little Brothers Chick Growing Mash, Little Brothers, Kalamazoo.

TABLE I. Eight Groups of Cockerels Fed Normal Diet—8 Weeks Experimental Period—*Without* Atherosclerotic Plaque Development.

Series 1 (38 birds)		Blood serum cholesterol				
		Not incubated		Incubated 30 hr at 37 to 38°C		
Atherosclerotic plaques (% area)		Total (mg %)	Free (mg %)	Free to combined form (mg %)	Free remaining (mg %)	% original free remaining after incubation
.0		104	27.2	22.5	4.7	17.3
.0		106	27.4	19.3	8.1	29.6
.0		100	26.6	18.0	8.6	32.3
.0		104	26.2	16.8	9.4	35.9
.0		94	24.5	15.1	9.4	38.4
.0		103	26.9	16.1	10.7	39.8
.0		104	26.0	15.0	11.9	44.2
.0		98	24.4	13.5	10.9	44.7
Mean values	.0	102	26.2	17.0	9.2	35.3
Range	—	94–106	24.4–27.4	22.5–13.5	4.7–11.9	17.3–44.7

relative balance between amounts of original free cholesterol combined and not combined upon incubation.

An outstanding incubation difference is the large increase above normal for uncombined free cholesterol in the severely atherosclerotic series. Values for the normal, mild spontaneous, and atherosclerotic series are 9, 14, and 67 mean mg% respectively. Original free cholesterol combined upon incubation shows small differences for the three series (17, 13, and 23 mean mg %). Corresponding approximate ratios of uncombined to combined upon incubation for the 3 series in the order named are .5, 1, and 3.

Statistical. Small increases in serum cholesterol mean values for normal compared with mild spontaneous series before incubation are not statistically significant. In contrast, all corresponding probability values

comparing normal with severe atherosclerotic, or the latter with mild spontaneous series, and in all possible interseries comparisons of extent of arterial plaques were highly significant, with $P < .001$. Probability values comparing normal with mild spontaneous series relative to (a) mg % original free cholesterol combined, (b) mg % and (c) % of original remaining after incubation are $> .02$, $> .001$, and $< .01$ respectively. Corresponding sets of values for comparisons of normal and mild spontaneous with severe series are .05, $< .001$, $< .01$, and $< .02$, $< .001$, $> .001$.

Summary. Relationship between serum free cholesterol not combined upon incubation and atheroma development has been studied using sera from normal and cholesterol-fed young cockerels. Original free cholesterol uncombined after incubation of serum from normal, mild spontaneous, and severely

TABLE II. Five Groups of Cockerels Fed Normal Diet—8 to 31 Weeks Experimental Periods—*With Low Degrees of Spontaneous* Atherosclerotic Plaque Development.

Series 2 (31 birds)		Blood serum cholesterol				
		Not incubated		Incubated 30 hr at 37 to 38°C		
Atherosclerotic plaques (% area)		Total (mg %)	Free (mg %)	Free to combined form (mg %)	Free remaining (mg %)	% original free remaining after incubation
.16		100	26.5	14.5	12.0	45.3
.20		123	29.7	15.8	13.9	46.8
.14		119	28.6	13.9	14.7	51.4
.20		105	26.2	11.6	14.6	55.7
.02		94	22.8	10.1	12.7	55.7
Mean values	.14	108	26.8	13.2	13.6	51.0
Range	.02–.20	94–123	22.8–29.7	15.8–10.1	12.0–14.7	45.3–55.7

TABLE III. Eight Groups of Cockerels Fed .5% Cholesterol Atherogenic Diet—8 Weeks Experimental Period—With Severe Atherosclerotic Plaque Development.

Series 3 (57 birds)		Blood serum cholesterol				
Atherosclerotic plaques (% area)		Not incubated		Incubated 30 hr at 37 to 38°C		
		Total (mg %)	Free (mg %)	Free to com- bined form (mg %)	Free remaining (mg %)	% original free remain- ing after incubation
14.1		219	59.5	25.8	33.7	56.6
20.1		297	76.1	26.5	49.6	65.2
19.6		416	104.6	35.1	69.5	66.4
10.8		325	82.0	24.6	57.4	70.0
26.8		314	84.5	21.0	63.5	75.1
18.0		456	121.7	23.7	98.0	80.5
19.1		396	102.1	14.7	87.4	85.6
16.6		328	90.2	11.8	78.4	86.9
Mean values	18.1	344	90.1	22.9	67.2	73.3
Range	10.8–26.8	219–456	59.5–121.7	35.1–11.8	33.7–98.0	56.6–86.9

atherosclerotic birds is 9, 14, and 67 mean mg % respectively. Amounts combined upon incubation show smaller differences (17, 13, and 23 mean mg %) for the 3 series. Corresponding ranges of per cent original free cholesterol not combined are 17 to 45 for normal, 45 to 56 for mild spontaneous, and 57 to 87% for severe cholesterol-induced atherosclerosis. Results of this study indicate that there is a relationship between serum free cholesterol not combined upon incubation and development of atheroma in young cockerels.

The author thanks Mr. Warren R. Lincoln for his technical assistance, and Dr. Harris D. Webster, of Pathology Research, for assistance in evaluating the arteries.

1. Sperry, W. M., *J. Biol. Chem.*, 1935, v111, 467.
2. Turner, K. B., McCormack, G. H., Jr., Richards, A., *J. Clin. Invest.*, 1953, v32, 801.
3. Morrison, L. M., Wolfson, E., Berlin, P., *Circulation*, 1950, v2, 479.
4. Caldwell, C. T., Suydam, D. E., *Proc. Soc. Exp. Biol. and Med.*, 1959, v101, 299.

Received March 1, 1961. P.S.E.B.M., 1961, v106.

Mitochondrial α -Glycerophosphate Dehydrogenase Activity of Juxtaglomerular Cells in Experimental Hypertension and Adrenal Insufficiency. (26514)

R. HESS AND A. G. E. PFARSE (Introduced by D. G. Scarpelli)

Department of Pathology, Postgraduate Medical School, London, England

It has been established in both man and experimental animals that the juxtaglomerular (JG) cells are related to formation of extractable renin in the kidney, that they respond to alterations of sodium metabolism and, possibly, participate in mechanisms maintaining systemic arterial pressure. The physiological state of these cells, which form part of the preglomerular arterioles, is usually assessed by estimating the amount of stainable cytoplasmic granules(1), which are believed to be secretory in nature and may contain renin

(2). The type of metabolic pathway operating in JG cells is unknown.

Using a histochemical technic, mitochondrial α -glycerophosphate dehydrogenase (GPD, Meyerhof-Green enzyme) can be demonstrated to be particularly active in the JG cells. This activity changes in the same direction as renin content in both experimental hypertension and adrenal insufficiency and seems to indicate one of the major metabolic steps which govern the functional state of the juxtaglomerular complex.

TABLE I. α -Glycerophosphate Dehydrogenase Activity* of Rat Kidney Mitochondria.

Addition	Cortex	Medulla
None	.37	.72
Amytal ($5 \times 10^{-3}\text{M}$)	.36	.70
Menadione (10^{-4}M)	1.50	4.10
" + amytal ($5 \times 10^{-3}\text{M}$)	1.44	3.90
" + antimycin A, $10 \mu\text{g/ml}$	1.53	4.30
" + dicoumarol (10^{-5}M)	.12	.24

* Expressed as μg of formazan/mg protein/min. (mean values of 2 exp.).

Materials and methods. A first group of 11 male albino rats (mean initial weight 158 g) were rendered hypertensive by unilateral clamping of the renal artery. Their blood pressure increased from 120 ± 2.9 mm Hg to 184 ± 7.4 mm Hg 36 days after operation. A second group of unilaterally nephrectomised animals (mean initial weight 106 g) were overdosed with desoxycorticosterone acetate (DOCA) and sodium chloride; after 36 days of treatment, systolic pressure had risen from 96 ± 1.8 mm Hg to 145 ± 5.1 mm Hg. Details of the experimental procedure have been reported(3). A comparable group of 6 animals were adrenalectomised and put on a sodium deficient diet for 7 days. Untreated animals served as controls.

For histochemical examination, 5 to 8 μ fresh frozen cryostat sections were used from slices of the middle parts of the kidneys which had been quenched in liquid oxygen. The sections, mounted on coverslips, were incubated for 30 minutes at 37° in a medium containing substrate, 10^{-1}M , (sodium L- α -glycerophosphate); 2-methyl-1,4-naphthoquinone (menadione), 10^{-4}M ; tris (hydroxymethyl) aminomethane-HCl buffer, 5×10^{-2} M, pH 7.4; 3-(4,5-dimethyl thiazolyl-2) 2,5 diphenyl tetrazolium bromide (MTT), $5 \times 10^{-3}\text{M}$; CoCl_2 , 10^{-3}M . For demonstration of lactate and malate dehydrogenase activity, menadione was omitted and the sodium salt of L-lactic acid or L-malic acid was used as substrate, in presence of diphosphopyridine nucleotide (DPN), $5 \times 10^{-3}\text{M}$.

In vitro, mitochondria from cortex and medulla of normal rat kidneys isolated in 0.25M sucrose and frozen-thawed, were incubated in the α -glycerophosphate medium at 37° and the cobalt-formazan formed was

measured spectrophotometrically at 660 m μ in ethyl acetate.

Results. The *in vitro* effect of some activators and inhibitors on specific GPD activity of cortical and medullary mitochondria is shown in Table I. Menadione is a more efficient primary electron acceptor than tetrazolium in this system and formazan production is increased 4- to 5-fold in its presence. Hydroquinone reduces tetrazolium non-enzymically(4). GPD is highly sensitive to dicoumarol but, in contrast to the menadione-dependent GPD of rat brain(5), it is not inhibited by amytal.

Distribution of GPD in normal rat kidney. High activity was found in the tubules of the outer medulla (descending and ascending limb) and in the cortical collecting ducts. Moderately strong activity was displayed by JG cells, distal convolutions (including the macula densa segment), vascular endothelium and medullary collecting duct epithelium. Both glomerular epithelium and interstitial cells were weakly active. No reaction occurred in the proximal convolutions or in vascular smooth muscle.

Renal hypertension. Five weeks after unilateral clamping of the renal artery, GPD activity was strongly enhanced in JG cells (Fig. 1). In the untouched contralateral kidney JG-cell activity was lower than normal and in some areas, decreased to zero (Fig. 2). These changes were more pronounced in outer cortical areas than in zones adjacent to the medulla.

DOCA and saline-induced hypertension. JG cells throughout the cortex were very low in activity and in many areas no GPD reaction could be obtained.

Adrenal insufficiency. Seven days after adrenalectomy an increased activity of GPD was observed in JG cells throughout the cortex. The appearance of the cells in adrenal insufficiency was very similar to that found in the ischemic kidney of animals bearing a Goldblatt clamp. In contrast to JG cells, GPD activity in the other renal structures was not significantly altered by the experimental procedures. After DOCA-saline overdosage, GPD activity was increased in col-

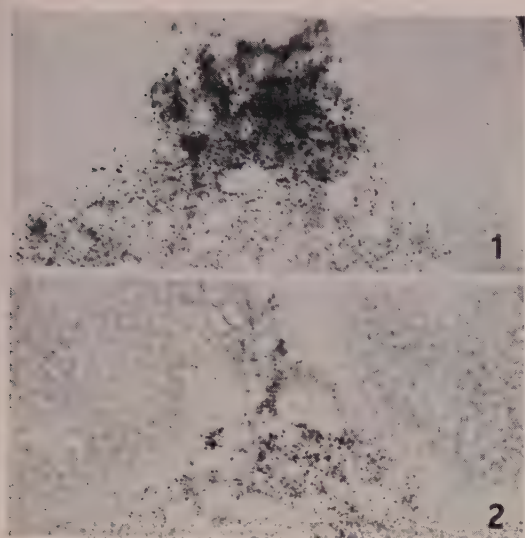


FIG. 1. High activity of mitochondrial GPD in JG cells (ischemic kidney from a rat bearing a Goldblatt clamp). 580 \times .

FIG. 2. Same animal as Fig. 1. Low GPD activity in JG cells of unclamped kidney. 580 \times .

lecting duct epithelium of the papilla in about half the animals tested.

Lactate and malate dehydrogenase activity in JG cells, in muscle cells of the afferent arteriole, and in cells of the glomerular tuft, was increased in areas showing hypertrophic vascular changes in hypertensive animals. However, no deviation from normal activity occurred in adrenal insufficient rats.

Discussion. McManus' concept that JG cells and the macula densa segment of the distal tubule form a functional unit (*viz.* the "juxtaglomerular complex") (6) is well supported by observations on the relationship of enzymic activity present in these structures. Although a different enzyme system is shown to prevail in the two parts composing the JG complex (glucose-6-phosphate dehydrogenase (G6PD) in macular cells (3,7,8) and mitochondrial GPD in JG cells), both kinds of activities have been demonstrated to change in the same way as renin content.

Metabolic activity of the macular segment seems to a large extent to depend on the function of the hexosemonophosphate (HMP) shunt which, by supplying reduced triphosphopyridine nucleotide, is linked to biological synthetic systems (9). The non-coenzyme linked GPD (10) on the other hand, partici-

pates in the α -glycerophosphate cycle (11) which, in certain tissues (insect flight muscle) provides the main energy-yielding mechanism. Histochemical proof for the existence of the complete GP-cycle is lacking, however, since we found it impossible to separate DPN-dependent cytoplasmic GPD activity (von Euler-Baranowski enzyme) from its mitochondrial counterpart by means of tetrazolium reduction. The equilibrium of the cytoplasmic GPD is shifted far toward GP (12). It may be significant that changes in GPD activity in JG cells were not accompanied by similar alterations of lactate dehydrogenase. Dissociation of these two activities together with low levels of lactate dehydrogenase has been found to occur in insect flight muscle biochemically (13) and histochemically (Hess and Pearce, unpublished). Furthermore, it is unlikely that the glycogen cycle is operating in JG cells. We were unable to demonstrate glucosan phosphorylase activity (14) in them, in contrast to muscle cells of the afferent arterioles which were strongly active.

If we postulate that triose phosphate isomerase is present in the JG complex, then glyceraldehyde-3-phosphate generated by the HMP pathway (9) could provide the dihydroxyacetone phosphate necessary for operation of the GP-cycle. A metabolic pathway of this sort might represent a biochemical link between the two major parts of the JG complex which have been shown recently to be in intimate communication by means of membranous structures (15).

Summary. Non-coenzyme linked α -glycerophosphate dehydrogenase activity has been demonstrated histochemically in juxtaglomerular cells of rats. Menadione, which increased the activity of rat kidney mitochondria 4- to 5-fold *in vitro*, was required as intermediate electron carrier when a thiazol substituted monotetrazole was used as final H-acceptor in tissue sections. α -Glycerophosphate dehydrogenase activity of juxtaglomerular cells increased both in ischemic kidneys from renal hypertensive rats and in kidneys from adrenal insufficient animals. Decrease in activity of juxtaglomerular cells was noted both in untouched kidneys from ani-

mals bearing a unilateral arterial clamp and in the renal cortex of rats overdosed with DOCA on a high sodium diet.

Tissues from hypertensive rats were kindly supplied by Dr. F. Gross, Pharmaceutical Department of CIBA, Ltd., Basel.

1. Hartroft, P. M., Hartroft, W. S., *J. Exp. Med.*, 1953, v97, 415.
2. Edelman, R., *Anat. Rec.*, 1960, v136, 185.
3. Gross, F., Hess, R., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v104, 509.
4. Slater, T. F., *Nature*, 1959, v183, 50.
5. Hess, R., Pearse, A. G. E., *ibid.*, in press.
6. McManus, J. F. A., *Lancet*, 1942, v2, 394.
7. Hess, R., Pearse, A. G. E., *Brit. J. Exp. Path.*, 1959, v40, 243.

8. Hess, R., Gross, F., *Am. J. Physiol.*, 1959, v197, 869.
9. Dickens, F., Glock, G. E., McLean, P., in *Regulation of Cell Metabolism. A CIBA Foundation Symposium*, London, 1959, 150.
10. Green, D. E., *Biochem. J.*, 1936, v30, 629.
11. Bücher, Th., Klingenberg, M., *Angew. Chemie*, 1958, v70, 552.
12. Young, H. L., Pace, N., *Arch. Biochem. Biophys.*, 1958, v75, 125.
13. Delbrück, A., Zebe, E., Bücher, Th., *Biochem. Z.*, 1959, v331, 273.
14. Takeuchi, T., *J. Histochem. Cytochem.*, 1956, v4, 84.
15. Oberling, Ch., Hatt, P.-Y., *Compt. Rend. Soc. Acad. Sci.*, 1960, v250, 929.

Received March 1, 1961. P.S.E.B.M., 1961, v106.

Trachoma Viruses Isolated in the United States.* 4. Infectivity and Immunogenicity for Monkeys. (26515)

CHANDLER DAWSON, ERNEST JAWETZ, PHILLIPS THYGESON AND LAVELLE HANNA
Francis I. Proctor Foundation for Research in Ophthalmology and Departments of Ophthalmology and Microbiology, University of California, Medical Center, San Francisco

Monkeys and apes have been the only laboratory animals useful in trachoma research. Baboons and apes have been more susceptible to experimental infection than the lower primates, but even in these animals the experimental disease, produced irregularly by transfer of conjunctival scrapings, has been equivocal—at best a mild follicular conjunctivitis without inclusions. The primate eye has shown more reaction to transfer of material from the closely related disease, inclusion conjunctivitis, developing an experimental disease often acute in onset and more closely resembling the human disease(1).

With egg-grown trachoma virus it became possible to produce regularly in rhesus and cynomolgus monkeys a clear-cut clinical disease with an exudate presenting a characteristic cytologic picture and with inclusion bodies in the conjunctival epithelial cells. It was soon apparent that isolates differed in their pathogenicity for these monkeys, and we have

reported experiments on the *qualitative* aspects of this difference as displayed by 2 strains of trachoma virus isolated in the United States(2,3). This report deals with attempts to *quantitate* the infectivity of these strains and to induce resistance to challenge infection.

Materials and methods. Viruses: Three strains of trachoma virus(4) and one strain of inclusion conjunctivitis virus(5) isolated in this laboratory were employed. Several yolk sac passage levels of each strain with the following egg LD₅₀ titers (ELD₅₀/ml) were used: Strain BOUR, YS3 to YS12, 10^{4.4} to 10^{6.4} ELD₅₀/ml; Strain ASGH, YS4 to YS8, 10^{5.8} to 10^{7.4} ELD₅₀/ml; Strain APACHE #1, YS8, 10^{6.4} ELD₅₀/ml; Strain IC-Cal 1 of inclusion conjunctivitis virus, YS9, 10^{5.6} ELD₅₀/ml. Stock virus was prepared, stored and titrated as described(6).

Monkey inoculations. For eye challenge, stock virus was diluted in skim milk. Small cotton swabs were soaked in the inoculum and rubbed back and forth 10 times in the upper and lower conjunctival fornices. An

*Supported by grants from Nat. Inst. Health, Burroughs, Wellcome Fd. and Res. Comm. of Univ. California.

estimated 0.1 ml of virus inoculum was thus placed onto the conjunctiva. For immunization, monkeys were used which had been infected in the past and had completely recovered, without any residual eye abnormalities. They were injected intramuscularly with 50% suspensions of crude infected yolk sac in broth containing $10^{6.2}$ to $10^{7.4}$ ELD₅₀/ml. Three injections of 0.8, 1.6 and 2.0 ml were given at 6 to 7-day intervals. Eight days after the third injection the monkeys were challenged with a conjunctival inoculum of 0.1 ml BOUR strain estimated to contain $10^{2.4}$ ELD₅₀/ml.

Recording of observations. The animals' eyes were examined at 2 to 4-day intervals with the aid of a focused bright light and a binocular loupe. The examiner washed his hands and flamed instruments after examining each monkey. All examinations were made by 2 of us (C.D. and P.T.) without knowledge of the nature of the inoculum or previous treatment of the monkeys. For each eye examination the following conjunctival changes were recorded on a scale of 0 (absent) to +++ (maximal intensity): hyperemia, discharge, infiltrate and lymphoid follicles. At most clinical examinations, conjunctival scrapings were taken with a platinum spatula, smeared on a glass slide, stained by Giemsa's method and studied for one-half hour each, to establish the cytologic picture and the presence of typical inclusion bodies.

Scoring of results. To quantitate observations and permit comparison of clinical impressions it was necessary to assign numerical values to the recorded data. Of the several conjunctival changes resulting from trachoma virus infection of the monkey eye, lymphoid follicles were the most suitable for quantitation. Their appearance, persistence, number and size could be estimated most accurately by different observers, and their development was highly specific for trachoma-inclusion conjunctivitis virus infection. To minimize inevitable subjective variation in the evaluations by the different observers on different days, the 3 highest follicle scores for both eyes of each monkey during 21 days of inoculation were pooled. By this method,

TABLE I. Dose-Infectivity Relationship of 2 Strains of Trachoma Virus in Cynomolgus Monkeys.

Titer, ELD ₅₀ /ml	No. monkeys positive/No. inoculated			
	BOUR strain clinical disease	Inclusion positive smears	ASGH strain clinical disease	Inclusion positive smears
$10^{5.8-6.7}$	3/3	3/3	6/6	5/6
$10^{5.4}$			4/5	1/5
$10^{4.4}$	2/2	2/2	5/5	4/5
$10^{3.4}$	4/4	4/4	2/2	0/2
$10^{2.4}$	4/5	3/5	2/2	0/2
$10^{1.4}$	2/2	1/2		

the highest total score obtainable was 18 (3 plus \times 2 eyes \times 3 observations). Values of Chi square were calculated using a modification for small numbers which gives more conservative values(7).

Results. Infectivity: The largest number of monkey infections were carried out with 2 strains of trachoma virus, BOUR and ASGH. The former was isolated from an acute initial infection in California, the latter from a Pakistani with a relapse of chronic trachoma. The infectivity of these 2 strains for cynomolgus monkey eyes is shown in Table I. On the basis of egg infectivity it can be seen that strain BOUR produces clinical disease more regularly and in higher dilution than strain ASGH. This strain difference is emphasized by the regular and abundant presence of inclusions in BOUR infections and their irregular and sporadic occurrence in ASGH infections. The infectivity of BOUR for cynomolgus eyes appears to be similar to that for embryonated eggs, as $10^{0.4}$ ELD₅₀ ($10^{1.4}$ ELD₅₀/ml) produced clinical "monkey trachoma" in 2 of 2 inoculated animals and inclusions in one of them. The data in Table I do not permit estimation of a "minimal infective dose" of either strain for the monkey eye in terms of egg infectivity. However, it is apparent that the average minimal "inclusion producing dose" is about $10^{0.5}$ ELD₅₀ of BOUR and more than $10^{3.0}$ ELD₅₀ for ASGH.

Severity of infection. Initial observations suggested that strain BOUR was not only able to infect monkey eyes with a smaller inoculum than ASGH but also produced regu-

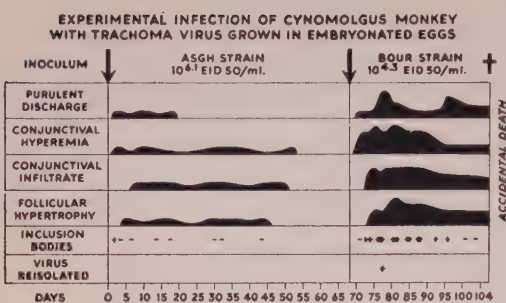


FIG. 1. Different clinical intensity of eye disease resulting from 2 strains of trachoma virus in the same monkey.

larly a more severe and prolonged disease of the eye. This strain difference is illustrated in Fig. 1, which shows the relatively mild disease caused by ASGH followed by a much more intense disease caused by BOUR, in spite of the smaller challenge inoculum employed with the latter strain. To substantiate this impression, follicle scores obtained for all rhesus and cynomolgus monkeys infected with comparable infective doses of these 2 strains are compared in Table IIA. By an arbitrary division of scores it was evident that 10 of 13 BOUR infections were above the half-maximum score, whereas only 2 of 19 ASGH infections fell into that range.

Repeated conjunctival infection. We have described elsewhere(2) that monkeys can be reinfected with the same or a different strain of trachoma virus when signs of the initial infection have subsided. Three such consecutive infections do not appear to modify response to subsequent challenge. In one attempt to superinfect *during* the active phase of experimental trachoma 2 monkeys were inoculated on the conjunctiva with 10^{5.7} ELD₅₀ BOUR virus within 4 weeks after

TABLE II. Follicle Scores as Index of Severity of Infection with Trachoma Viruses.

	Score			P
	0-9	10-18	Chi ²	
A. Diff. between strains				
BOUR	3*	10*	12.72	.001
ASGH	17	2		
B. Effect of immunization				
Control	1	3	5.61	.02
Immunized	10	0		

* No. of monkeys in score category; see text for details of experiments.

their inoculation. The animal originally infected with ASGH, however, developed additional disease manifestations with numerous inclusions. The suggestion that some degree of "infection-immunity" might be engendered by the homologous but not the heterologous strain will have to be confirmed in larger numbers of monkeys.

Effects of parenteral virus administration.

In a group of 14 rhesus monkeys we attempted to determine whether repeated large intramuscular doses of live trachoma virus could modify the course of subsequent eye infection. As described above, 10 monkeys received between 10⁷ and 10⁸ ELD₅₀ of live virus of different strains intramuscularly in 3 weekly injections. Four additional monkeys were kept as controls. Eight days after last intramuscular injection all animals were challenged with a conjunctival inoculum of 10^{1.4} ELD₅₀ strain BOUR (10^{2.4} ELD₅₀/ml). The results are presented in Tables IIB and III.

TABLE III. Effect of Immunization on Rhesus Monkeys to Challenge with 10^{1.4} ELD₅₀ BOUR Virus (10^{2.4} ELD₅₀/ml).

Immunizing strain	No. infected/No. challenged	Inclusion positive smears	Mean follicle scores
Control	4/4	4	11.5
BOUR	4/4	1	5.0
ASGH	2/2	1	8.25
APACHE #1	2/2	1	8.0
IC Cal 1	2/2	0	5.25

All challenged monkeys manifested some signs of infection, but immunized animals had less clinical activity and few developed inclusion bodies. The follicle scores of immunized monkeys were significantly lower ($p = 0.02$) than those of controls. The details of the experiment (Table III) indicate that only 3 of 10 immunized animals, but all 4 controls, developed demonstrable inclusions, suggesting some limitation of viral multiplication in the immunized animals. The average follicle score for monkeys immunized with any one of 3 strains of trachoma virus (BOUR ASGH, APACHE #1) and one strain of inclusion conjunctivitis virus (IC Cal 1) was lower than for the controls. The most marked reduction in average follicle

score applied to monkeys immunized with BOUR or IC Cal 1. However, numbers are too small to permit interpretation.

Discussion. The results presented here confirm the impression(2) that rhesus and cynomolgus monkeys may serve as useful alternative hosts for study of viruses of the trachoma-inclusion conjunctivitis group. In contrast to the highly variable results obtained with direct "tissue transfer" of conjunctival scrapings from human trachoma to the monkey eye(1), the inoculation of egg-grown viruses resulted in predictable and reproducible disease. There was a general correlation between amount of virus in the inoculum (expressed as ELD₅₀/ml) and rapidity of onset of the disease as well as intensity of clinical signs (hyperemia, discharge, infiltrate and number and size of follicles). However, with increasing dilution of viral inoculum, clinical signs were less well defined. Thus the number of ELD₅₀ constituting a minimum "monkey infective dose" could not be accurately estimated on the basis of clinical signs alone.

The more severe the experimental monkey trachoma infection, the greater was the number of inclusion bodies found in conjunctival scrapings. While the microscopic search for inclusion bodies is tedious and time consuming, it lends a measure of objectivity to the other criteria of monkey infection. By such combined assessment it could be unequivocally determined that in terms of ELD₅₀ strain BOUR was at least 100 times more infectious for monkeys than strain ASGH, *i.e.*, 100 times more ASGH virus was required to produce a clinical "take" in monkeys with inclusions.

By the "follicle score" method proposed here, differences in severity of infection may be quantitated with some confidence. Why BOUR should regularly produce a more severe disease in monkeys than ASGH, however, remains uncertain. Strains isolated in China(8), Saudi Arabia(9), Gambia(10), and Taiwan(11) tend to produce a mild disease akin to that produced with ASGH. The patient from whom the BOUR strain was isolated in California had no known contact with

trachomatous persons, but did develop corneal changes and a pannus entirely typical of trachoma. The greater virulence of monkey infection with BOUR virus is a remarkable strain characteristic, since in the past, viruses of inclusion conjunctivitis have caused a more severe disease of monkey eyes than trachoma. The BOUR strain appears to occupy a unique position in the trachoma-inclusion conjunctivitis group which requires further study.

Like trachoma in humans, experimental trachoma in monkeys did not give rise to demonstrable resistance of the eye to reinfection with the same or a different strain of virus. Very large amounts of virus injected repeatedly intramuscularly did alter the clinical response significantly, suggesting that systemic "immunity" may at times play a role. We have not yet attempted to measure "species-specific" antibodies(12) in these monkeys and cannot comment on their possible role. It will be of interest to examine the effect of parenteral virus administration on the characteristic extended clinical disease and the protracted presence of the virus in the human conjunctiva.

Summary. Three strains of trachoma virus and one strain of inclusion conjunctivitis virus isolated in the United States gave reproducible disease upon inoculation into eyes of rhesus or cynomolgus monkeys. Among several clinical criteria follicle scores were most suitable for quantitation of eye disease, and microscopic demonstration of inclusions in conjunctival scrapings served as additional criterion of infection. Significant differences were demonstrated in the infectivity of 2 strains for the monkey eye. Repeated eye infection failed to modify the response to reinfection. Repeated parenteral administration of live virus in large quantities significantly diminished disease from subsequent challenge infection but failed to induce solid immunity.

1. Thygeson, P., Crocker, T. T., *Am. J. Ophthalm.*, 1955, v42, 76.

2. Thygeson, P., Dawson, C., Hanna, L., Jawetz, E., Okumoto, M., *Am. J. Ophthalm.*, 1960, v50, 907.

3. Hanna, L., Thygeson, P., Jawetz, E., Dawson, C., *Science*, 1959, v130, 1339.

4. Hanna, L., Jawetz, E., Thygeson, P., Dawson, C., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v104, 142.
5. Hanna, L., Zichosch, J., Jawetz, E., Vaughan, D. G., Jr., Thygeson, P., *Science*, 1960, v132, 1660.
6. Jawetz, E., Hanna, L., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v105, 207.
7. Hill, A. B., *Principles of Medical Statistics*, New York, Oxford Univ. Press, 1955, p141.
8. T'ang, F. F., Chang, H. L., Huang, Y. T., Wang, K. C., *Chin. Med. J.*, 1957, v75, 429.
9. Murray, E. S., Bell, S. D., Hanna, A. T., Nichols, R. L., Snyder, J. C., *Am. J. Trop. Med. and Hyg.*, 1960, v9, 116.
10. Collier, L. H., Sowa, J., *Lancet*, 1958, v1, 993.
11. Grayston, J. T., Wang, S., Johnston, P. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1960, v103, 596.
12. Woolridge, R. L., Jackson, E. B., Grayston, J. T., *ibid.*, 1960, v104, 298.

Received January 27, 1961. P.S.E.B.M., 1961, v106.

Friends of the Society

Our members are requested to note the following list of Friends. We wish to express our thanks to each of them. Their contributions help materially in meeting the very sharp increase in costs of publication.

FRIENDS

Abbott Laboratories, Research Division	Chas. Pfizer and Co., Inc.
American Cancer Society	Pitman Moore Co.
American Cyanamid Co.	Riker Laboratories, Inc.
American Tobacco Company	The Rogoff Foundation
Ayerst Laboratories	Damon Runyon Memorial Fund
Bristol Laboratories, Inc.	Sandoz Pharmaceuticals
Burroughs Wellcome Co., Inc.	Schering Corporation
Ciba Pharmaceutical Products, Inc.	G. D. Searle and Co.
Eaton Laboratories, Division of the Norwich Pharmacal Co.	Sharp and Dohme, Division of Merck & Co., Inc.
Foundation for Microbiology	Smith Kline and French Laboratories
Louis and Bert Freedman Foundation	Smith, Miller & Patch, Inc.
Geigy Pharmaceuticals, Division of Geigy Chemical Corporation	Squibb Institute for Medical Research
Hoffmann-LaRoche, Inc.	Sterling-Winthrop Research Institute
Eli Lilly and Co.	G. N. Stewart Memorial Fund
Lipotropic Research Foundation	U.S. Vitamin Corporation
Mead Johnson & Co.	The Upjohn Co.
Merck Institute for Therapeutic Research	Wallace Laboratories, Division of Carter Products, Inc.
The Wm. S. Merrell Co.	Warner-Chilcott Laboratories
Miles Laboratories, Inc.	The Wilson Laboratories
Ortho Pharmaceutical Corporation	Wyeth Laboratories
Parke Davis and Co.	Anonymous

AUTHORS' INDEX

VOLUME 106

(The numerals indicate the page)

- Abe, T. 506.
 Abinanti, F. R. 466.
 Adams, T. 862.
 Ambrose, A. M. 656.
 Anderson, J. T. 555.
 Anderson, R. R. 567.
 Andrus, C. H. 809.
 Angelakos, E. T. 762.
 Antoniadis, H. N. 831.
 Arms, R. J. 130.
 Artman, M. 610.
 Ashikawa, J. K. 547.
 Atkinson, W. B. 348.
 Averich, E. 523.
 Bader, J. P. 311.
 Bagdonas, A. A. 90.
 Bahler, R. C. 383.
 Baird, J. M. 362.
 Baker, B. L. 65.
 Baker, R. D. 603.
 Ballantyne, D. L., Jr. 182.
 Banerjee, S. 63.
 Bang, B. G. 516.
 Bang, F. B. 516.
 Banks, I. S. 212.
 Barron, A. L. 648.
 Bates, R. W. 435.
 Battisto, J. R. 725.
 Bavetta, L. A. 738, 741.
 Beckfield, W. J. 263.
 Beigelman, P. 484.
 Bekesi, G. 300.
 Bekhor, I. 741.
 Bekierkunst, A. 610.
 Belanger, L. F. 19.
 Bellet, S. 514.
 Beraldo, W. T. 29.
 Bercovici, B. 147.
 Berg, R. B. 581, 614.
 Bergen, S. S., Jr. 173.
 Bernhart, F. W. 588.
 Bernheimer, A. W. 776.
 Berry, L. J. 855.
 Bezman, A. 450.
 Bickerton, R. K. 834.
 Bier, O. G. 29.
 Biglieri, E. G. 806.
 Bishop, D. W. 24.
 Bittner, J. J. 303, 844.
 Blackmore, W. P. 681.
 Blumberg, A. G. 867.
 Blume, L. 533.
 Bohner, B. 632.
 Bold, E. J. 133.
 Bollman, J. L. 438.
 Boris, A. 708.
 Borman, A. 231.
 Botticelli, C. R. 887.
 Bouroncle, B. 654.
 Bowser, E. N. 664.
 Braun, W. 748.
 Bray, R. E. 255.
 Brennan, D. M. 231.
 Brent, R. L. 523.
 Brindle, S. A. 880.
 Brody, P. 298.
 Brody, T. M. 816.
 Brookreson, A. D. 567.
 Brown, E. R. 303, 844.
 Brown, E. S. 93.
 Browning, H. C. 558.
 Brzezinski, A. 147.
 Buckley, J. P. 834.
 Bueker, E. D. 373.
 Buffett, R. F. 426, 455.
 Bullard, R. W. 341.
 Burns, J. J. 157.
 Burt, R. L. 330.
 Butler, W. T. 383.
 Butterfield, J. L. 259.
 Buxton, R. W. 162.
 Bydeman, S. 537.
 Byers, S. O. 796.
 Cain, S. M. 7.
 Caldwell, C. T. 893.
 Calvert, D. N. 816.
 Cannon, J. A. 133.
 Capps, J. H. 540.
 Carbone, J. V. 526.
 Carey, J. B., Jr. 800.
 Carlisle, H. N. 654.
 Carr, C. W. 652.
 Chai, C. K. 521.
 Chang, R. S. 149.
 Chanock, R. M. 466.
 Chen, G. 632.
 Cheng, H. F. 93.
 Christophe, J. 405.
 Clark, R. H. 65.
 Cohen, P. P. 170, 492.
 Cohen, R. B. 309.
 Cohen, S. N. 677.
 Cole, M. B. 692.
 Connor, W. E. 378.
 Converse, J. M. 182.
 Cook, M. K. 466.
 Cooper, W. G. 801.
 Corbin, A. 841.
 Cornatzer, W. E. 194.
 Cornfeld, D. 717.
 Corrigan, Jr., J. J. 420.
 Corte, G. 327.
 Costa, E. 693.
 Costello, J. 708.
 Coulson, E. J. 40.
 Covert, S. V. 729.
 Covill, R. W. 603.
 Crandell, R. A. 542.
 Cronkite, E. P. 49.
 Cummings, J. R. 470.
 Damm, H. C. 820.
 Dannenburg, W. N. 330.
 Davidson, C. S. 642.
 Davidson, W. D. 514.
 Dawson, C. 898.
 De Eds, F. 656.
 Delchamps, E. E. 317.
 Denton, A. D., Jr. 444.
 De Ropp, R. S. 696.
 De Vries, A. 462.
 Dicks, M. 93.
 Dienst, R. B. 400.
 Doberneck, R. C. 552, 877.
 Drake, J. W. 755.
 Drapiewski, V. A. 523.
 Drewes, P. A. 295.
 Driscoll, T. B. 181.
 Dubach, U. 136.
 DuPont, J. R. 113.
 Eichhorn, J. 153, 354, 781.
 Eichwald, E. J. 441.
 Ells, H. A. 607.
 El Zayat, S. 803.
 Emerson, C. P. 313.
 Emery, C. E., Jr. 267.
 Endres, J. G. 370.
 Engle, R. L., Jr. 365, 652.
 Ercoli, N. 787.
 Ershoff, B. H. 306, 605.
 Evans, J. S. 350.
 Fanska, R. 526.
 Fedor, E. J. 275.
 Feinberg, L. J. 514.
 Feinstein, M. 781.
 Feldman, H. A. 736.
 Felsenfeld, O. 175.
 Fennell, A. 450.
 Fine, J. 394.
 Fine, M. B. 339.
 Finkelstein, R. A. 481.
 Finland, M. 73, 78.
 Fisher, B. 275.
 Fisher, H. 61.
 Flathers, R. 346.
 Fleisher, G. A. 283.
 Forsblad, K. R. 350.
 Foulk, W. T. 438.
 Frank, E. D. 394.
 Frantz, I. D., Jr. 800.
 Freed, S. C. 35.
 Freedman, H. H. 495.
 Friedell, G. H. 313.
 Friedman, L. 570.
 Friedman, M. 796.
 Friedman, M. H. F. 645.
 Friedman, S. M. 10.
 Fullmer, H. M. 157.
 Furth, J. 455.
 Fuson, R. B. 441.
 Gal, E. M. 295.
 Galasso, G. 669.
 Gallagher, N. I. 127.
 Ganaway, J. R. 542.
 Ganong, W. F. 806.
 Gantt, C. L. 27.
 Garcia, J. F. 585.

- Garrison, M. M. 435.
 Gaylor, J. L. 576.
 Gedalia, I. 147.
 Geesey, C. N. 625.
 Geiringer, E. 752.
 Gertler, M. M. 109.
 Ghosh, P. K. 63.
 Gibbs, G. E. 368.
 Gilbert, D. L. 550.
 Giuffre, N. A. 880.
 Glassman, P. M. 762.
 Glick, D. 359.
 Golden, M. 354.
 Goldenberg, H. 867.
 Gomoll, A. W. 196.
 Good, R. A. 472, 476, 480, 572, 875.
 Goodman, J. W. 822.
 Gouze, M. L. 4.
 Gower, M. M. 708.
 Grace, J. T., Jr. 423.
 Grande, F. 555.
 Greenberg, A. D. 666.
 Greenman, D. L. 459.
 Greenspan, R. H. 540.
 Griffen, W. O., Jr., 101, 552.
 Griffith, D. R. 448, 873.
 Griminger, P. 61.
 Grindlay, J. H. 438.
 Grodsky, G. M. 526.
 Gross, L. 890.
 Grossekreutz, D. C. 794.
 Grupp, E. 219.
 Gump, D. 24.
 Gustafsson, G. T. 277.
 Habel, K. 722.
 Hagan, D. Q. 127.
 Hale, H. B. 597.
 Hale, W. H. 486.
 Hales, M. R. 540.
 Halkerston, I. D. K. 153, 781.
 Hall, F. G. 199.
 Hamilton, T. R. 836.
 Hamparian, V. V. 717.
 Hanna, L. 898.
 Hanson, L. E. 755.
 Harper, A. E. 184.
 Harrington, P. A. 270.
 Hartsook, E. W. 57.
 Hashim, S. A. 173.
 Hatch, E. 136.
 Haurowitz, F. 93.
 Hazelwood, R. L. 851.
 Hazzard, W. R. 839.
 Hechter, O. 153, 354, 781.
 Heggeness, F. W. 812.
 Henderson, W. J. 664.
 Herbener, G. H. 348.
 Herman, Y. F. 542.
 Hershberger, T. V. 57.
 Hess, M. 420.
 Hess, R. 895.
 Hilker, D. M. 121, 335.
 Hill, J. 812.
 Hill, J. M. 444.
 Hilleman, M. R. 717.
 Himeno, K. 45.
 Hiramoto, R. 661.
 Hirata, M. 263.
 Hisaw, F. L., Jr. 887.
 Hodak, J. A. 420.
 Hodgson, G. 766.
 Hoffman, B. F. 90.
 Hoffman, J. G. 423.
 Hoggan, M. D. 212.
 Hokfelt, B. 537.
 Hollander, F. 629.
 Honska, W. L. 181.
 Hopkins, T. F. 140.
 Hori, S. H. 545.
 Horita, A. 32.
 Horowitz, M. I. 629.
 Horwitz, D. 118.
 Hosoda, T. 506.
 Houck, J. C. 145.
 Howell, D. S. 317.
 Howell, E. V. 673.
 Hsia, D. Y. 533.
 Huang, I. 533.
 Hunsaker, W. G. 498.
 Hunter, J. H. 350.
 Hurley, L. S. 343.
 Hutchings, R. H. 368.
 Hyde, P. M. 142.
 Ingram, R. L. 52.
 Jacob, R. A. 145.
 Jacobs, D. H. 1.
 Jansen, V. 248, 251.
 Jasmin, R. 327.
 Jawetz, E. 898.
 Jean, P. 408.
 Jeanes, J. K. 432.
 Jeanrenaud, B. 405.
 Jerushalmy, Z. 462.
 Johnson, G. A. 124.
 Johnson, P. C. 181.
 Johnson, W. 327.
 Jones, R. S. 177, 673.
 Jumper, J. R. 52.
 Kaeberle, M. L. 755.
 Kampschmidt, R. F. 870.
 Kaneko, T. 506.
 Kao, K. Y. T. 121, 335.
 Karzon, D. T. 648.
 Kawahata, A. 862.
 Keele, D. K. 168.
 Keller, S. 49.
 Kelley, G. W. 592.
 Kelman, H. 875.
 Kemp, J. G. 498.
 Kennan, A. L. 170.
 Kent, H. N. 710.
 Kent, J. F. 729.
 Kessel, J. F. 409.
 Ketler, A. 717.
 Keys, A. 555.
 Khanade, J. M. 764.
 Khazan, N. 579.
 Kilham, L. 825.
 Kim, H. 409.
 Kind, L. S. 734.
 Kirkman, H. N. 607.
 Kirsh, D. 685.
 Kistler, C. R. 124.
 Kizer, D. E. 790.
 Klein, J. R. 288.
 Klein, R. 784.
 Koch, M. B. 136.
 Kocsis, J. J. 659.
 Koelle, G. B. 625.
 Kohn, A. 462.
 Komiyama, T. 45.
 Korngold, L. 365.
 Kostos, V. J. 659.
 Kowalewski, K. 300.
 Kraintz, L. 113.
 Kritchevsky, D. 704.
 Kummerow, F. A. 370.
 Kunkel, H. G. 291.
 Lacey, D. E. 790.
 Laferte, R. O. 391.
 Lamy, F. 160.
 Lange, K. 13.
 Lange, R. D. 127.
 Lansing, A. I. 160.
 Lassen, N. A. 743.
 Lauber, J. K. 871.
 Lawton, A. H. 692.
 Lazarov, E. 147.
 Leake, N. H. 330.
 Lee, N. D. 684.
 Lehrman, D. S. 298.
 Leikin, S. L. 286.
 Leonard, A. S. 552, 877.
 Leonard, S. L. 839.
 Lerner, L. J. 231.
 Leskowitz, S. 357.
 Levenson, S. M. 267.
 Levine, L. 502.
 Levinthal, J. D. 426, 455.
 Levy, R. P. 214.
 Lewis, W. P. 409.
 Li, Y. T. 398.
 Lian, T. S. 207.
 Liebelt, R. A. 55.
 Liepins, H. 149.
 Lipchuck, L. M. 470.
 Litwack, G. 700.
 Liu, F. T. Y. 521.
 Loken, M. K. 239.
 Longarini, A. E. 450.
 Longley, J. B. 743.
 Longmire, W. P., Jr. 133.
 Loomis, T. A. 490.
 Lustgraaf, E. C. 441.
 Luther, H. G. 486.
 McCarthy, J. M. 127.
 McClelland, L. 717.
 McCloskey, R. V. 85.
 McCluer, R. H. 124.
 McDonald, T. P. 107.
 McGavack, T. H. 121, 335.
 McGinnis, J. 871.
 McLean, R. A. 855.
 McPherson, S. E. 13.
 Ma, S. 409.
 Macchi, I. A. 324.
 Mac Donald, J. B. 394.
 MacDonald, R. A. 809.
 Mackie, J. E. 642.
 Madrazo, A. 732.

- Makino, S. 545.
 Mandel, B. 772.
 Maqsood, M. 104.
 Margolish, M. 149.
 Martin, G. R. 157.
 Martinez, C. 472, 476, 480, 572, 875.
 Masson, G. M. C. 315.
 Matsumoto, T. 545.
 Maung, M. 70.
 Mead, J. F. 4.
 Meffers, R. B., Jr. 597.
 Meites, J. 104, 140.
 Mellett, L. B. 221.
 Mengel, G. D. 350.
 Metzger, J. F. 212.
 Michaelis, M. 162.
 Migicovsky, B. B. 19.
 Mihich, E. 97.
 Mikasa, A. 315.
 Milch, R. A. 68.
 Miller, L. L. 270.
 Miraglia, G. J. 333.
 Mirand, E. A. 423, 501.
 Moore, K. E. 816.
 Morgan, H. R. 85, 311.
 Morter, R. L. 600.
 Morton, D. L. 727.
 Mosser, D. G. 239.
 Mourad, S. 570.
 Muller-Eberhard, H. J. 291.
 Munoz, J. 70.
 Muschel, L. H. 622.
 Musser, A. E. 350.

 Nading, L. K. 88.
 Nastuk, W. L. 502.
 Nath, M. C. 42, 764.
 Nazario, M. 492.
 Neter, E. 97.
 Neuman, R. E. 857.
 Nichols, A. V. 547.
 Nicoloff, D. M. 101, 552, 877.
 Niemann, W. H. 542.
 Nilsson, I. M. 277.
 Nimni, M. E. 738.
 Ninomiya, H. 162.
 Norris, G. R. 288.
 Noval, J. J. 748.

 O'Brien, K. D. 851.
 O'Connell, P. W. 848.
 O'Day, P. 741.
 Odell, T. T., Jr. 107.
 Oh, J. O. 413.
 Oliver-Gonzalez, J. 710.
 Ooyama, H. 387.
 Orth, R. D. 339.
 Osada, Y. 248, 251.
 Otken, L. B., Jr. 52.
 Otto, W. K. 673.

 Packer, J. T. 16.
 Palczuk, N. C. 748.
 Palmer, J. 498.
 Palmerio, C. 394.
 Panner, B. J. 16.
 Paronetto, F. 216.
 Payne, R. W. 189.

 Pearse, A. G. E. 895.
 Pechet, G. 809.
 Pedreira, L. 365.
 Peizer, L. R. 772.
 Peric-Golia, L. 177.
 Perkins, E. G. 370.
 Perlman, D. 880.
 Perry, J. H. 55.
 Petersdorf, R. G. 234.
 Phillips, G. B. 192.
 Pierce, W. A., Jr. 207.
 Pilecci, V. J. 684.
 Pipberger, H. V. 130.
 Plescia, O. J. 748.
 Popper, H. 216.
 Pothapragada, S. 359.
 Potter, H. D. 511.
 Premachandra, B. N. 818.
 Prentice, T. C. 423, 501.
 Pressman, D. 164, 661, 769.
 Prigmore, J. R. 594.
 Pscheidt, G. R. 693.

 Ratner, A. 140.
 Recant, L. 136.
 Rehnborg, C. S. 547.
 Reid, A. F. 432.
 Reilly, C. M. 717.
 Reiner, L. 732.
 Reiter, J. H. 194.
 Rennels, E. G. 362.
 Renold, A. E. 405.
 Reynolds, W. M. 486.
 Ribble, J. L. 234.
 Richardson, G. S. 357.
 Richardson, J. A. 259.
 Richart, R. 829.
 Riemer, W. 317.
 Rigby, P. G. 313.
 Robbins, D. J. 656.
 Roberts, A. N. 93.
 Roberts, F. E. 692.
 Robinson, W. S. 115.
 Roizman, B. 320.
 Roffler, S. 734.
 Rosanoff, E. I. 563.
 Rosen, H. 267.
 Rosenberger, C. R. 223.
 Rosenkrantz, H. 391.
 Rosenthal, M. S. 581, 614.
 Rubini, J. R. 49.
 Rugh, R. 219.
 Rustigian, R. 757.

 Sackner, M. A. 514.
 Sadler, W. A. 558.
 Salzano, J. 199.
 Sampliner, J. 214.
 Sandberg, A. A. 831.
 Sandberg, H. 514.
 Sanyal, P. N. 639.
 Saslaw, S. 654.
 Saunders, P. R. 639.
 Schaffner, F. 216.
 Schluenderberg, A. E. 320.
 Schoenfield, L. J. 438.
 Schottelius, B. A. 841.
 Schultz, G. A. 870.
 Schwartz, L. L. 776.

 Schweinburg, F. B. 394.
 Scotch, D. S. 324.
 Scott, G. T. 88.
 Scott, M. L. 635.
 Scully, E. 153, 354.
 Segal, H. L. 270.
 Selye, H. 408.
 Seto, T. 545.
 Shafer, W. G. 205.
 Shannon, I. L. 594.
 Shapiro, F. 472, 476.
 Sharp, D. G. 669.
 Shaver, D. N. 648.
 Shaw, C. W. 223.
 Shellhamer, R. H. 93.
 Shepard, C. C. 685.
 Sherman, W. C. 486.
 Sherrod, T. R. 196.
 Shetlar, M. R. 398.
 Shrager, P. 700.
 Shulman, J. A. 234.
 Shutze, J. V. 871.
 Silver, R. T. 365.
 Silverstein, E. 381.
 Siqueira, M. 29.
 Sjoerdsma, A. 118.
 Skelton, F. R. 142.
 Slaunwhite, W. R., Jr. 831.
 Small, M. D. 450.
 Smith, C. W. 212.
 Smith, J. M. 472, 572.
 Smith, K. O. 669.
 Smith, R. L. 530.
 Smulow, J. B. 757.
 Snedeker, E. H. 696.
 Snyder, F. 202.
 Snyder, I. S. 836.
 Snyder, J. L. 341.
 Soffer, L. J. 1.
 Spain, D. M. 452.
 Speer, R. J. 444.
 Speir, R. W. 403.
 Speirs, R. S. 248, 251.
 Spink, W. W. 242, 280.
 Spolter, P. D. 184.
 Sreter, F. A. 10.
 Stallmann, F. W. 130.
 Staple, E. 704.
 Starnes, W. R. 883.
 Steinman, H. G. 227.
 Stephens, N. 202.
 Stevens, H. 40.
 Stevens, R. W. 729.
 Stewart, R. B. 666.
 St. George, S. 35.
 Stoewsand, G. S. 635.
 Stokes, J., Jr. 717.
 Stone, N. H. 101, 877.
 Stormont, J. M. 642.
 Straus, E. K. 617.
 Stuckey, J. H. 90.
 Sugioka, K. 794.
 Sulman, F. G. 579.
 Sultz, B. M. 495.
 Sutfin, D. C. 511.
 Swisher, S. N. 629.
 Synek, J. H. 27.

 Tamanoi, I. 661, 769.

- Tanabe, Y. 45, 506.
Tannenbaum, S. 533.
Tanyol, H. 645.
Tedeschi, L. G. 865.
Teller, R. 484.
Terasaki, P. I. 133.
Terrill, K. 239.
Thomas, A. N. 727.
Thurau, K. 714.
Thygeson, P. 898.
Timiras, P. S. 343.
Tomarelli, R. M. 588.
Toy, B. L. 865.
Trabold, N. 629.
Trager, R. 21.
Turner, C. W. 448, 567, 818, 820, 873.
Tye, M. 757.
Tytell, A. A. 857.

Underdahl, N. R. 592.
Usher, P. 784.
Uzgiries, V. 664.

Vadalkar, K. 42.
Van Arsdel, P. P., Jr. 255.
Van Dyke, D. C. 585.

Van Heyningen, H. E. 37.
Van Itallie, T. B. 173.
Van Tienhoven, A. 803.
Van Wart, C. A. 113.
Veilleux, R. 408.
Vener, J. 480.
Venerose, R. S. 90.
Vick, J. A. 242, 280.
Von Kaulla, K. N. 530.
Vrbanovic, D. 732.

Wachstein, M. 13.
Wakim, K. G. 283.
Wallmark, G. 73, 78.
Wang, S. S. 736.
Wangensteen, O. H. 101, 552, 877.
Warfield, M. 466.
Weber, L. J. 32.
Webster, J. 168.
Weiss, H. S. 61.
Weissman, D. 772.
Weissman, I. 441.
Welsch, J. A. 708.
Wende, N. M. 400.
Whitehouse, M. W. 704.
Whitsett, T. L. 189.

Widmann, W. D. 540.
Williams, G. A. 664.
Williams, M. W. 603.
Williams, R. H. 339.
Winnik, H. Z. 579.
Wohlfromm, M. 219.
Wolf, R. O. 362.
Wong, D. 466.
Wood, L. 49.
Woods, K. R. 652.
Woods, L. A. 221.
Woodward, J. M. 333.
Woolley, D. E. 343.
Wortis, R. P. 298.
Wotiz, H. H. 887.
Wright, E. B. 387.
Wright, L. D. 21.
Wright, P. A. 346.

Yagi, Y. 164, 661, 769.
Yamamoto, R. S. 381.
Yang, Y. T. 413.
Young, G. A. 592.

Zamcheck, N. 450.
Zarrow, M. X. 459.
Zeppa, R. 794.

SUBJECT INDEX

VOLUME 106

(The numerals indicate the page)

- Acetazolamide**, effect on passage of protein. 113.
 -reserpine combination, antihypertensive action. 470.
 ventilatory effect. 7.
- Acetylcholine**, microbioassay. 502.
- Acid**, amino, sperm metabolism. 803.
 in brain, drugs. 696.
 metabolism. 184.
 tyrosine, metabolism in thermal injury. 267.
- aminocaproic, epsilon-, in shock. 242.
- DNA**, antigenicity. 748.
 experimental mammary growth. 567.
 synthesis in inflammatory cells. 251.
- fatty, atherosclerosis. 61.
 free, cerium fatty liver. 202.
 incorporation into plasma lipids. 339.
 non-esterified, control in plasma. 330.
 of mouse lipids, lipoproteins. 547.
 homogenetic, in alcaptonuria. 68.
- nucleic, metabolism. 149.
 organic, excretion, Ca absorption after indigestible carbohydrate. 588.
 sialic, metabolism. 124.
- Adenosine**, cardiovascular effects. 762.
 triphosphate, relationship to RNA. 21.
- Adrenal** cortex, regeneration, response to stress. 142.
 response to bilateral carotid constriction in hypophysectomy. 806.
 secretion, acetylcholine. 324.
 D-xylose permeability, ACTH. 354.
 glands, lipids, sex. 381.
- Age**, connective tissue protein. 335.
 liver phospholipid metabolism. 194.
 response to erythropoietin. 585.
- Air**, expired, CO₂ measuring device. 692.
- Alcaptonuria**, HGA binding collagen. 68.
- Amebiasis**, hemagglutination test for entamoebae. 409.
- Amino**-hexose-reductones, toxicity. 656.
- Amonia** metabolism, human adult fetal liver. 170.
- Anaphylaxis**, passive, antiserum. 40.
 cutaneous, Schultz-Dale, Ovary test. 29.
 Schultz-Dale. 70.
- Angiotensin**, induction of hypertension. 834.
- Antibiotic**, penicillins, antistaphylococcal activity. 78.
 biochemical comparison. 227.
- Antibody**, antilung. 661.
 antikidney. 769.
 anti-lymphosarcoma. 164.
 chicken atheroma. 383.
 fluorescent, reactions against MTA. 303.
 stainability. 685.
 staining technic. 52.
 treponemal test. 729.
 glomerulonephritis. 13.
 group A streptococcal, detection. 207.
 hepatic diseases. 216.
 lupus, erythematosus. 622.
 production, neonatal guinea pig. 24.
 response, blood diseases. 654.
 to homografts. 133.
 vaginal. 617.
- Antigen**, autoradiographic localization. 93.
 specific, from animal parasites. 710.
- Antiserum**, routes of administration, passive anaphylactic sensitization. 40.
- Arteriosclerosis**, experimental. 383.
- Arthritis**, alcaptonuria patients. 68.
- Atherosclerosis**, fatty acids, corn sterol. 61.
 aorta lipids. 4.
 serum incubation, free cholesterol. 893.
- Bacterium**, myco-, fluorescent antibody. 685.
phlei, lactic dehydrogenase inhibition. 610.
tuberculosis H37Rv, growth *in vivo*. 610.
- Pasteurella tularensis*, energy metabolism. 333.
- Staphylococcus*, activity of penicillins. 78.
 effect of extract on tumor. 97.
 extracellular proteins. 776.
 new phages. 73.
- Streptococcus*, group A, antibody detection. 207.
 production of streptolysin S. 836.
- Bentonite**, absorption of endogenous pyrogens. 234.
- Bile** acids, cholesterol-4-C¹⁴. 177.
 inhibition of vit. B₁₂ absorption. 181.
- Bilirubin** monoglucuronide, extrahepatic. 438.
- Blood**, anemia, hemolytic, *Leptospira pomona* antigen, antibody. 600.
 circulation in renal medulla. 743.
 diseases, antibodies. 654.
 erythrocytes, *in vivo* survival using sodium chromate⁵¹. 313.
 inhibition of P³² loss by oxalate. 432.
 manganese uptake. 288.
 reduction, gonadal influences. 1.
 erythropoietin, age. 585.
 dose-response. 127.
 in rabbit plasma, hypoxia. 501.
 flow, vasopressin. 511.
 group substances, gastric secretion. 629.
 infusion, sampling apparatus. 498.
 leucocytes, measles virus. 581.
 leukemia, fibroblast culture. 614.
 mouse viral. 426.
 thymus. 455.
 radioyttrium colloids. 444.
 rats, induction with mouse leukemia virus. 890.
 lipase inhibition. 192.
 lymphocytes, glycogen content. 286.
 in granuloma pouch. 263.
 metabolism, radioactive phosphate, oxalate. 432.
 plasma erythropoiesis stimulating factor. 766.
 platelets, life span. 107.
 myxoviruses. 462.
 prolactin inactivation. 140.
 serum proteins, electrophoretic patterns. 373.
 thermolabile, hemolytic activity. 291.
 thrombus production, intra-arterial, with polyethylene tubing. 796.

- Bone**, vit. D, rickets. 19.
- Brain**, amino acids, drugs. 696.
lesions with gold-thioglucoase. 55.
Pasteur reaction in shock. 162.
- Calcium** absorption, vitamin D, cortisone. 664.
- Cancer**, azo-dye, development, effect on serine dehydrase, cystathionine synthetase. 790.
lymphosarcoma and mycoplasma (PPLO). 673.
prostate, treatment with stilbestrol diphosphate. 327.
- Carbohydrate**, dietary, source, effect on survival time. 605.
indigestible, Ca and acid excretion. 588.
metabolism, domestic birds. 459.
- Carbon** dioxide in human cell culture. 149.
measurement in expired air. 692.
tetrachloride, liver injury, liver extract treatment. 645.
- Cataract**, experimental, lens sodium, potassium concentrations. 812.
- Cell** culture, serum and embryo extract replacement. 857.
Earle's L, vaccinia virus adsorption. 669.
fibroblasts, culture from leukemic, nonleukemic patients. 614.
HeLa, hydrocortisone effect in herpes. 666.
homologous lymphoid, transplant, and erythematous disease. 725.
human, culture. 149.
inflammatory, DNA synthesis. 251.
qualitative, quantitative response. 248.
mast, succinic dehydrogenase. 359.
renal, feline, viral susceptibility. 542.
- Cerebrospinal** fluid, effect of acetazolamide. 113.
- Chlordiazepoxide**, endocrine effects. 708.
- Cholera** antiphage, human sera. 175.
- Cholesterol**, biosynthesis inhibition by Triparanol (MER-29). 452.
effect of dietary oil on liver content. 800.
oleyl alcohol in pancreatic extract. 848.
-4-C¹⁴, conversion into bile acids. 177.
synthesis, negative feedback mechanism. 136.
- Colchicine**, taurine excretion. 659.
- Collagen** binding homogentisic acid solutions. 68.
connective tissue. 335.
distant dermal, response to local inflammation. 145.
excess ascorbic acid, biosynthesis. 741.
- Cystic** fibrosis, sweat thiocynate. 368.
- Desmosterol**, local tissue reaction in rabbits. 452.
- Diabetes**, insulin and microsomes. 115.
- Diet**, amino acids, growth 184.
carbohydrate source, effect on survival time. 605.
fattening of lambs, cattle, steroidal sapogenins. 486.
fiber, pectin, serum cholesterol. 555.
high fat, radiation toxicity. 306.
oils, effect on liver cholesterol content. 800.
protein, ascorbic acid, effect on collagen biosynthesis. 741.
- Dihydrotychsterol** overdosage, sensitization by thallium. 408.
- Diuretics**, benzhydroflumethiazide and hydrochlorothiazide, lack of extrarenal effect. 681.
- Drug**, anticonvulsant properties of 1-(1-phenylcyclohexyl) piperidine. 632.
excitants, depressants, brain amino acids. 696.
metabolism, dephosphorylation of psilocybin. 32.
- Electrocardiography**, computer, automatic screening. 130.
- Electrolytes**, nitrogen excretion, rat growth. 597.
in connective tissue. 317.
resuscitation of isolated heart. 196.
- Electrophoretic** studies, chicken serum, egg yolk. 506.
- Electroshock** in manganese deficiency. 343.
- Embryo**, chick, immune response. 182.
experimental congenital malformations. 523.
x-irradiation. 219.
- Encephalomyelitis**, Venezuelan equine, demonstration by immunofluorescence. 212.
- Endocrine** effects of chlordiazepoxide. 708.
relationships, pituitary-ovary. 346, 362.
- Entamoebae**, hemagglutination test. 409.
- Enzyme**, cholinesterase, in neurohypophysis. 625.
fibrinolytic factors in crevicular fluid. 277.
glucose-6-phosphate dehydrogenase, erythrocytic, assay. 607.
glutamic-oxalacetic transaminase in serum after liver injury. 283.
hyaluronidase, allergic reactions. 734.
leucinamidase in swine influenza, parasite complexes. 592.
lipoprotein lipase in lymph. 378.
monoamine oxidase inhibitor. 118.
phosphamidase in rat uterus. 348.
phosphoglucomutase in muscle, vitamin E-deficient chicks. 839.
proelastase in Goose-fish. 160.
reaction to tocopherol. 391.
succinic dehydrogenase, in mast cells. 359.
trypsin, measurement of activity. 239.
- Eye**, avian, light effect. 871.
- Fat** composition in rat, effect of oxidized oil. 370.
indigestion, induction by bile acid sequestrant. 173.
- Fibrinolysis** in fluid from gingival crevice. 277.
induction by urea derivatives. 530.
- Flectol H**, hepatic injury. 16.
- Fluorine**, placental transfer. 147.
- Fungus**, *Candida albicans*, active immunization. 570.
- Gastric** mucosa, effect of hypophysectomy. 65.
secretion, blood group substances. 629.
- Glomerulonephritis**, experimental. 13.
- Glucose** and plasma non-esterified fatty acids. 330.
cycloacetoacetate, glutathione depletion in necrosis. 42.
gold-thio-, brain lesions. 55.
transport in adipose tissue, amino acids. 484.
- Gonads**, erythrocyte reducing capacity. 1.
- Growth**, organ size, effect of SC-6582, Ro2-7239. 57.

- Guanethidine**, cardiovascular effect, catechol amines. 259.
- Heart**, cardiovascular effects of adenosine. 762.
computer in electrocardiographic diagnosis. 130.
conducting system, recording from chronic intracardiac electrodes. 90.
interarterial coronary anastomoses, neonatal pigs. 732.
isolated, resuscitation. 196.
normal, failing, energy metabolism. 109.
ouabain effect on potassium. 639.
temperature gradients, hypothermia in ventricular fibrillation. 275.
- Hemagglutination** test, *Entamoeba*. 409.
- Heparin**, distribution, excretion. 490.
effect on lymph, plasma. 378.
- Heterosis**, prenatal, x-irradiation. 219.
- Histamine**, release by meperidine. 794.
from rat mast cells. 255.
- Homogenates**, RNA, ATP. 21.
- Hormone**, ACTH, and adrenal D-xylose permeability. 354.
and insulin, effect on AIB accumulation. 153.
effect on sex ratio. 752.
synthesis and pituitary RNA. 420.
aldosterone, regulation of secretion. 806.
corticosterone, plasma, in regenerating adrenal glands. 142.
cortisone, intestinal calcium absorption. 664.
effects on α -aminoisobutyrate accumulation in muscle. 781.
epinephrine production, effect of 2-deoxy-D-glucose in rat. 537.
estrogen, in human plasma. 831.
therapy in posterior hypothalamic lesions, rat. 841.
uterine sensitivity, age. 521.
gonadotropin secretion in lactation. 558.
neural control. 298.
growth, human, latex agglutination by reaction with antiserum. 168.
hydrocortisone analogues, serum, urine steroid levels. 594.
insulin and ACTH, effect on AIB accumulation. 153.
effect on scurvy. 63.
melanocyte stimulating, release by tranquilizers. 88.
progesterone, effect on frog ovulation. 346.
16 α , 17 α , dihydroxy-, derivatives. 231.
prolactin, immunoassay. 214.
inactivation by blood. 140.
serotonin, in starvation and thiamine deficiency. 295.
sex, in invertebrates. 887.
in peptic ulcer. 101.
stilbestrol diphosphate, enzymic hydrolysis. 327.
testosterone, localization of anabolic effects. 738.
thyrotropin, blood levels in mice with pituitary tumors. 435.
thyroxine, urea cycle enzymes, regenerating liver. 492.
mammary gland growth. 873.
metabolism. 818.
treatment, tissue oxygen consumption. 300.
vasopressin, blood flow, pressure. 511.
hydrogen ion concentration, muscle calcium, magnesium. 550.
- Hyperglycemia**, glucagon-induced, modification. 851.
- Hypertension**, action of acetazolamide-reserpine. 470.
MO-911. 118.
experimental, mitochondrial α -glycerophosphate dehydrogenase in juxtaglomerular cells. 895.
guanethidine, cardiovascular effect. 259.
induction by angiotensin. 834.
- Hypotension**, hemorrhagic, renal pressor substances. 315.
- Hypothalamus**, posterior, lesions, estrogen therapy. 841.
- Hypothermia**, cardiovascular reflex activity. 199.
catechol amines. 816.
ventricular fibrillation. 275.
- Hypoxia**, effect on plasma erythropoietin, rabbit. 501.
righting ability, pain threshold. 341.
renal Na-reabsorption, O₂-uptakes. 714.
- Immune** response, chick embryo. 182.
- Immunofluorescence**, Venezuelan equine encephalomyelitis in tissue culture. 212.
- Immunology**, autoimmunization. 357.
chicken serum, egg yolk. 506.
differences, lymphosarcoma and normal rat lymph nodes. 164.
prolactin. 214.
- Iodide**, intestinal absorption. 450.
- Irradiation**, gamma, effect on blood cholinesterase. 603.
- Kala azar**, serum protein. 365.
- Kidney**, countercurrent exchange, blood circulation. 743.
effect on aorta electrolytes. 35.
graft in hemorrhagic hypotension. 315.
Na-reabsorption, O₂-uptake. 714.
- Lactation**, electrical induction in rats. 104.
gonadotropin secretion. 558.
- Leishmaniasis**, chemotherapy. 787.
- Liver**, antibodies. 216.
biliary obstruction and regeneration. 809.
bilirubin monoglucuronide, extrahepatic, in experimental hepatectomy. 438.
carbon tetrachloride injury, liver extract treatment. 645.
cholesterol content. 800.
disease, serum fatty acids. 642.
effect of 1,2-dihydro-2,2,4-trimethyl-quinoline. 16.
fatty, after plasma free fatty acids. 202.
human, ammonia detoxication. 170.
injury, 2 GOT in serum. 283.
ketogenesis, effect of hydrolyzed glucose cycloacetoacetate. 764.
mitochondria, oxidation of ergosterol. 704.
necrosis, glucose cycloacetoacetate. 42.
phospholipid metabolism, age. 194.
rat, glycogen in shock. 162.
regenerating, effect of thyroxine on urea cycle enzymes. 492.

- Lung**, antibody localization. 769.
in radio-labelled antibodies. 661.
- Lupus** erythematosus, concentration of normal antibodies. 622.
- Lymph**, lipoprotein lipase. 378.
- Malaria** parasites, staining. 52.
- Mammary** gland, effect of steroids. 820.
growth, DNA. 567.
normal growth in rat. 448.
thyroxine effect on growth. 873.
secretion, electrical induction. 104.
- Manganese** deficiency, electroshock. 343.
- Mecholyl** test, catechol amine excretion. 867.
- Mercaptide**, sulfobromophthalein, biosynthesis with glutathione. 526.
- Mercaptopurine**, 6-, effect on homograft. 727.
- Morphine**, N-C¹⁴-methyl-, metabolism. 221.
- Mucous-ciliary** system, internal dehydration. 516.
- Muscle** activity, effect of pitressin. 10.
calcium, magnesium, effect of pH. 550.
effects of corticoids, insulin, epinephrine on α -aminoisobutyrate accumulation. 781.
potassium, effect of cooling. 787.
rat, glycogen in shock. 162.
vitamin E deficiency, phosphoglucomutase. 839.
- Mycoplasma** (PPLO) and lymphosarcoma. 673.
- Nephrosis**, rat, cholesterol synthesis. 136.
- Nerve**, activity, node membrane current, effect of potassium. 387.
- Nitrogen**, electrolyte excretion, rat growth. 597.
- Oil**, oxidized, effect on fat composition, rat. 370.
- Ouabain**, heart potassium. 639.
- Ovary**, ascorbic acid depletion after LH. 362.
Thio-TEPA, gonadotropin. 189.
- Oxygen** consumption, effect of hormones. 300.
- Pancreas**, effect of quinidine. 700.
proelastase localization in Goose-fish. 160.
- Parabiosis**, anemia-polycythemia. 441.
- Peptic** digestion, vascular factor. 552.
- Phenothiazine** derivatives, MSH release. 88.
- Phenylketonuria**, experimental, metabolism of 5-hydroxy-indole compounds. 533.
- Pitressin**, cationic exchange. 10.
- Pituitary**-adrenal cortex axis, effect of reserpine. 579.
effect of transplantation on ovarian ascorbic acid. 362.
on gastric mucosa. 65.
gonadotropin secretion. 298.
neurohypophysis, cholinesterase localization. 625.
RNA and ACTH production. 420.
- Placenta**, fluorine transfer. 147.
radioautographic studies of trophoblast. 829.
- Plasma**, binding of estrogen. 831.
- Potassium** excretion, effect of DOC and thiazide. 27.
muscle. 784.
nerve activity, ionic currents. 387.
- Pregnancy**, embolic trophoblast. 865.
- Protein**, Bence-Jones, binding of calcium. 652.
effect on vitamin A utilization. 635.
extracellular, of *Staphylococci*. 776.
replacement in rat connective tissue. 121.
- Psilocybin**, dephosphorylation. 32.
- Pyrogens**, endogenous, absorption by bentonite. 234.
- Quinidine**, effect on pancreas. 700.
- Radiocolloids**, yttrium hydroxide-Y-90, yttrium phosphate-Y-90. 444.
- Rare** earths and fatty liver. 202.
- Reserpine**, effect on pituitary-adrenal cortex axis. 579.
5HT interactions. 693.
in peptic ulcer. 877.
- Rheumatoid** sera, euglobulins. 883.
reaction with rabbit gamma globulin. 823.
- Runt** disease in tolerant mice. 572.
- Scurvy**, effect of 3-methylcholanthrene. 157.
insulin treatment. 63.
- Serum** cholesterol concentration, diet fiber, pectin. 555.
free, and atheroma. 893.
fatty acids, liver disease. 642.
glycoproteins, utilization. 398.
lipids, fatty acid incorporation. 339.
protein in kala azar. 365.
- Sex**-histocompatibility, size in survival of homografts. 476.
multiparous mice. 480.
ratio, effect of ACTH. 752.
- Shock**, endotoxin, effect of epsilon-aminocaproic acid. 242.
hydralazine with metaraminol, hydrocortisone. 280.
hemorrhagic, bacterial invasion. 394.
Noble-Collip, drum, rat, metabolic changes. 162.
- Skin** grafts, hamster cheek pouch, auto-, homo-, hetero-. 677.
homografts, acquired tolerance induced with blood, adult mice. 875.
adult mice. 472.
effect of 6-mercaptopurine. 727.
size, sex-histocompatibility. 476.
multiparous mice, sex-histocompatibility. 480.
serum antibodies. 133.
human, oral mucosa, cultivation. 757.
- Sodium** 22, kinetics, excretion rates. 514.
- Spermatozoa**, effect of amino acids. 803.
- Spleen** cell injections, homologous, acquired tolerance in adult mice. 472.
- Staining**, hematein, neoplastic tissues. 545.
- Steatorrhea**, experimental, in man. 173.
- Steroid** and phenanthrene compounds, growth. 57.
levels after hydrocortisone analogues. 594.
mammary gland growth. 820.
metabolism, domestic birds. 459.
- Steroidal** sapogenins for lambs, cattle. 486.
- Sterol** biosynthesis, rat skin. 576.
ergo-, oxidation by liver mitochondria. 704.
- Streptolysin** S, effect of mouse passage on production. 836.
- Sweat** gland distribution, racial variations. 862.
thiocyanate in cystic fibrosis. 368.
- Thallium** acetate, sensitization to DHT overdosage. 408.

- Thiouracil**, effect on thyroidal I¹³¹. 45.
- Thrombus**, intra-arterial, experimental production. 796.
- Thymidine**, tritiated, oral absorption. 49.
- Thymus**, mouse, viral leukemia. 426, 455.
- Thyroid**, effect of thiouracil. 45.
function, reaction to sulfadiazine. 37.
- Tissue**, adipose, glucose transport, effect of amino acids. 484.
metabolism, ACTH tumors. 405.
chemistry, L-gulonolactone oxidase activity. 309.
connective, collagen response to inflammation. 145.
electrolytes, sponge biopsy. 317.
protein and age. 335.
replacement. 121.
culture, antibiotic, use of Fungizone®. 880.
duck hepatitis virus. 755.
effect of Dilantin Sodium analogues. 205.
growth of arthropod-borne viruses. 223.
human skin, oral mucosa. 757.
plastic Petri dish. 801.
viral infection in L cells. 85.
- Tocopherol**, effect on enzymes, glycogenesis. 391.
- Toxin**, endo-, bacterial, effect on plasma iron in rats. 870.
body temperature changes. 855.
modification of lethality by zymosan. 495.
- Toxoplasmosis**, epidemiology. 400.
- Trachoma**, immunization, monkeys. 898.
- Tranquilizers**, MSH release. 88.
- Triiodothyronine-I¹³¹**, radiopurity, T-3 test. 684.
- Trophoblast**, embolic, in peripheral circulation during pregnancy. 865.
- Tumor**, adrenocorticotrophic, mice, and adipose tissue. 405.
effect of staphylococcal extract. 97.
inhibition *in vivo* by nucleoside. 350.
mouse mammary, agent, fluorescent microscopy. 303.
genetic variations. 844.
polyoma virus, resistance. 722.
- Ulcer**, peptic, effect of reserpine. 877.
sex hormones. 101.
- Urine**, deoxyribonuclease measurement, indole reaction. 270.
- Uterus**, estrogen sensitivity, age influence. 521.
phosphamidase in rat. 348.
- Venography**, portal, by retrograde hepatic flushing. 540.
- Virus**, arthropod-borne, cell culture. 223.
chloroform sensitivity. 736.
ECHO 22 and 23, cytopathology. 648.
electron microscopic counting. 669.
Friend, strain specificity, effect on spleen. 423.
hepatitis, in tissue culture. 755.
herpes, hydrocortisone effect on HeLa cells. 666.
leukemia, mouse. 426.
pathogenicity for rats. 890.
thymus. 455.
measles, hemagglutination, hemadsorption. 563.
in mitotic HEP-2 cells. 320.
propagation in leucocyte suspensions. 581.
Mengo, salt concentration, infectivity. 402.
myxo-, interaction with human blood platelets *in vitro*. 462.
para-influenza 3, human and bovine strains. 466.
Newcastle disease, endotoxin effect. 481.
nitrogen mustard effect on viral toxic reaction. 413.
polio, isolation technic. 772.
polyoma, immunity, resistance to transplanted tumors. 722.
psittacosis, latent infection. 85, 311.
rat, infection of hamsters. 825.
respiratory syncytial, laboratory findings in 109 cases. 717.
susceptibility, feline renal cells. 542.
trachoma, monkey infectivity. 898.
vaccinia, neutralized, adsorption by Earle's L cells. 669.
- Vitamin A**, effect of protein on utilization. 635.
B₁₂, inhibition of absorption. 181.
C, metabolism in thermal injury. 267.
D, calcium adsorption. 664.
in rickets. 19.
E deficiency, D- α -tocopherol and glycogenesis. 391.
phosphoglucosmutase in skeletal muscles. 839.
- X-Radiation**, embryo. 219.
high fat diets. 306.



